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THE UNIVERSITY OF ALBERTA

MODULATION OF ADIPOCYTE MEMBRANE COMPOSITION AND INSULIN ACTION IN NORMAL AND DIABETIC STATES BY DIETARY FAT

by

© CATHERINE JANE FIELD

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (NUTRITION AND METABOLISM)

EDMONTON, ALBERTA

FALL 1988

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TO MY MOTHER AND FATHER FOR THEIR LOVE AND ENCOURAGEMENT

ABSTRACT

Nutritionally complete semi-synthetic diets differing in the content and composition of fat were fed to control and streptozotocin-induced diabetic rats to assess if a relationship exists between dietary fat-induced alterations in the plasma membrane and membrane mediated insulin action. The effect of dietary fat was examined in terms of its influence on the following: 1. The lipid composition of the adipocyte plasma membrane; 2. The composition of stored lipids in the adipocyte; 3. Insulin binding; and 4. Insulin stimulated glucose metabolism.

Dietary fat influenced the content and fatty acyl composition of the adipocyte plasma membrane. Increasing the polyunsaturated to saturated fatty acid ratio (P/S) of the diet increased the content of polyunsaturated and reduced the content of monounsaturated fatty acids in both the adipocyte plasma membrane and stored lipids. High fat diets (20% w/w), compared to low fat diets (10% w/w), were associated with a higher content of phosphatidylethanolamine and phosphatidylinositol and a lower content of sphingomyelin in membranes. The diabetic state altered both the essential fatty acid and monounsaturated fatty acid content of adipocyte lipids and phospholipids.

Increasing the P/S ratio in a high fat diet enhanced insulin binding to control cells and increased insulin stimulated glucose transport, glucose oxidation and glucose incorporation into lipids in both diabetic and control cells. Low fat diets providing a high P/S ratio, compared to a low P/S ratio, improved insulin-stimulated glucose oxidation and lipogenesis by diabetic cells.

This thesis demonstrates that transitions in dietary fat intake, similar to those consumed by segments of the North American population, alter the structural lipids of the epididymal adipocyte plasma membrane and are associated with changes in insulin mediated cellular action. In support of the hypothesis that changes in the adipocyte plasma membrane microenvironment play a role in modulation of cellular insulin action, a direct positive relationship was established between dietary fat-induced membrane alterations and insulin binding. Although the physiological implications of these findings remain to be established,

the metabolic benefits of current dietary recommendations to replace a portion of saturated fats with polyunsaturated fats appear justified for both normal subjects and individuals affected with insulin resistant disorders such as diabetes.

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I. REGULATION OF INSULIN BINDING AND ACTION

A. INTRODUCTION

The objective of this thesis was to demonstrate that a relationship exists between dietary fat intake, membrane lipid composition and insulin action. It was hypothesized that, in both the normal and diabetic states, changes in the content and compostion of fat in the diet will alterin the epididymal adipocyte: 1. The lipid content and plasma membrane; and 2. Insulin stimulated functions of insulin binding, glucose transport and intracellular glucose metabolism.

Insulin regulates the synthetic phase of energy metabolism in cells by promoting glucose and amino acid uptake, lipogenesis, glucose oxidation and synthesis of protein and glycogen and by inhibiting lipolysis and proteolysis. Insulin action on target cells is initiated by binding to a specific plasma membrane receptor. Much progress has been made in the identification, characterization and isolation of the insulin receptor. These studies (Hedo & Simpson, 1985; Gammeltoft, 1984; Fujita-Yamaguchi, 1984; Roth & Taylor, 1982) have demonstrated that the receptor is subject to dynamic regulation by a variety of metabolic, hormonal and environmental factors. Resistance to the action of insulin plays a central role in many disease states, including diabetes and obesity. Insight into the causes and significance of insulin resistance in these disorders may aid in developing effective strategies for disease intervention.

B. INSULIN RECEPTOR

Structure

The insulin receptor is an integral membrane glycoprotein composed of two distinct subunits derived from a single chain polypeptide precursor (Hedo & Simpson, 1985). The α (M_r=135,000) and β (M_r=95,000) subunits are linked and assembled into a disulfide linked heterodimer ($\alpha_2\beta_2$) (Fujita-Yamaguchi, 1984). The α subunit is believed to contain the

insulin binding site while the β subunit is a transmembrane protein displaying tyrosine specific protein kinase activity and autophosphorylation of specific tyrosine residues (Ullrich et al., 1985).

Binding

The major method to study insulin receptor binding is to measure *in vitro* the interaction of radioactive labelled hormone with receptors on whole cells or membrane-rich factions of cells (Roth & Taylor, 1982). Utilizing this method, it has been clearly demonstrated that insulin binding to its plasma membrane receptor is rapid, reversible and site specific for insulin (Kahn et al., 1974; Gammeltoft, 1984). Although there is continual debate in the literature as to how to analyze ligand binding data, Scatchard plots are invariably used for the initial analysis of thermodynamic equilibrium binding data obtained from studies of reversible ligand-receptor binding (Scatchard, 1949). In this analysis, ratio of the concentraton of receptor-bound ligand to free ligand versus the concentration of bound ligand is plotted over a range of free ligand concentrations such that, when linear, the slope determines binding affinity and the intercept on the abscissa represents the number of binding sites. Bound hormone in this equation refers to that specifically bound, calculated by subtracting the amount of radiolabeled ligand remaining bound in the presence of excess unlabeled ligand from the total amount of radiolabelled ligand bound to receptors. Inherent in Scatchard analysis are these assumptions: thermodynamic equilibrium exists; binding is not disturbed when the bound ligand is separated from free; and the reaction is reversible. All of these assumptions appear to be reasonable in the case of insulin receptor binding (Gammeltoft, 1984). The major objection to the use of Scatchard analysis is centered around the interdependency of the axis coordinates that tend to amplify errors in measurement (Lefkowitz & Michel, 1983; Kahn et al., 1974; Gammeltoft, 1984). Analysis may be simplified in the future by the use of a computer assisted analytical approach that directly apply laws of mass action to analyze binding data by nonlinear least squares curve fitting programs (Lefkowitz & Micheal, 1983).

Insulin-receptor interactions in most cell populations and fractions are complex and characteristic of a curvilinear Scatchard plot (Gammeltoft, 1984). A nonlinear plot could be interpreted to be due to the presence of heterogeneous populations of binding sites or to negative cooperative interactions amongst a homeogeneous class of receptors. The general consensus in the literature has been that binding is of the negative-cooperative type, confirming earlier work by DeMeyts et al. (1973) that the dissociation rate of receptor-bound insulin was enhanced *in vitro* when dissociation was induced by dilution in presence of excess insulin. Recently, however, this method of interpreting negative cooperativity has been challenged (Helmerhorst, 1987).

The negative cooperative binding characteristic of the insulin receptor has been interpreted physiologically as an effective buffer against large oscillations in plasma insulin levels (Gammeltoft, 1984). Because insulin action is related to receptor occupancy, negative cooperativity would maintain insulin sensitivity at low insulin concentrations but attenuate an excessive cellular response at higher concentrations.

The amount of insulin bound to a particular cell or membrane preparation is a function of the receptor concentration, its affinity for the ligand and the concentration of the ligand. Receptor concentration depends on both the rates of receptor synthesis and degradation. Insulin receptors are in a highly dynamic state and half life has been estimated in a variety of cell types to be 4-10 hours (Lane, 1981; Kahn et al., 1974; Gammeltoft, 1984; Heidenreich et al., 1985). However, there is limited information on the biological fate and regulatory mechanisms involved in the metabolism of the insulin receptor. Insulin bound to the plasma membrane of rat adipocytes is rapidly translocated by receptor-mediated endocytosis process into at least two intracellular compartments (Sonne & Simpson, 1984). Insulin-receptor degradation appears to take place intracellularly in lysosomes with some receptors being recycled back to the plasma membrane (Heidenreich et al., 1985). Degradation increases with hormone occupancy (De Meyts et al., 1976) and may involve different mechanisms than those involved in the basal turnover of unoccuppied receptors (Heidenreich et al., 1985). Although the functional role of this process in not completely understood,

potential functions include: regulation of cell surface receptor number (Knutson et al., 1983); termination of insulin action by delivery of insulin to intracellular sites of degradation (Gammeltoft, 1984); or mediation of insulin action (Sonne, 1987; Jochen & Berhanu, 1987).

Several molecular mechanisms have been suggested to mediate insulin binding to cellular receptors. Early studies suggested that the receptor behaves as if composed of at least two functional components, an affinity regulator and a binding component (Harmon et al., 1983). The binding site has now been identified as the α-chain of the receptor located outside the cell and the affinity regulator as the β-subunit that transverses the plasma membrane (Hunter, 1985). The dimers of the insulin receptor have a reduced affinity for insulin compared to the intact receptor, suggesting that the disulfide bonds linking the two dimers may play a role in the ligand recognition function of the receptor (Boni-Schnetzler et al., 1987). Recently it was demonstrated that the insulin receptor exists in one molecular mass specie but in three interconvertible redox forms, which may bind and recognize insulin differently and it was hypothesized that factors within the membrane may be involved in the interconversion of these species (Yip & Moule, 1983). Studies utilizing photoaffinity-labelling of hepatic plasma membranes suggests that a component which is either part of, or closely associated with, the insulin receptor may regulate its affinity for insulin (Haynes et al., 1986). Recently it was observed that the α- and β-subunits of the receptor incorporate fatty acyl chains during post-translation modification (Hedo et al., 1987), suggesting that fatty acid acylation may play a role in the interaction and function of the receptor in the membrane.

Measurement of insulin binding has facilitated rapid advances in the study of the insulin receptor. It is now quite clear that both the number and properties of the receptor are dynamically modulated. Although the insulin receptor has been structurally characterized, mechanisms by which the binding event is regulated are not clear.

C. INSULIN ACTION

To understand the role of insulin in normal and pathophysiological states, it is necessary to assess the interaction of insulin with its target tissues. A biological dose curve, where a biological function is measured over a wide range of *in vitro* insulin concentrations, is commonly used to assess insulin action and to postulate the mechanisms of insulin resistance when insulin action is altered. Decrease in insulin receptors will lead to a shift to the right in the insulin-biologic function dose response curve with no decrease in maximal insulin action. This shift is termed a decrease in insulin sensitivity (Kahn, 1978; Flier, 1983). A pure postreceptor defect should lead to reduction in insulin action at all insulin concentrations, including those where the response is maximized and this is termed a decrease in insulin responsiveness (Kahn, 1978; Flier, 1983). However, because the precise cellular mechanisms of insulin action are not well understood it is possible that certain postreceptor defects could also alter insulin sensitivity. In addition, a combination of receptor and postreceptor defects will decrease both insulin sensitivity and responsiveness (Kahn, 1978; Flier, 1983).

Although the role of insulin in promoting glucose metabolism is most often studied, insulin exerts a wide variety of effects at the cellular level including activating or inactivating cytoplasmic and membrane enzymes, altering protein synthesis and DNA synthesis while influencing the processes of cell growth and differentiation (Flier, 1983).

Glucose Transport

The stimulation of hexose transport is an important and early effect of insulin and has been studied in detail in adipocytes and to some extent in skeletal muscle (reviewed by Simpson & Cushman, 1986). It has been suggested that insulin sensitive tissues have a distinctive type of glucose transporter (Wang, 1987). Recently, it was demonstrated that within an insulin sensitive tissue, glucose transporters exist as heterogeneous species, differing in the degree of glycosylation (Wang, 1987; Matthaei et al., 1987).

Because of its marked sensitivity to insulin, the hexose transport system in rat adipocytes has been intensively studied. Presently, there are several techniques available to

measure transport of nonmetabolizable sugars or sugar analogs in adipocytes. D-glucose, 2-deoxyglucose and 3-0-methylglucose all share a common glucose transport system (Pedersen & Gleimann, 1981). Due to the rapid metabolism, difficulties arise in measuring D-glucose uptake directly (Whitesell & Gliemann, 1979; Pedersen & Gleimann, 1981). The nonmetabalizable sugar 2-deoxyglucose is rapidly transported and phosphorylated by hexokinase but not further metabolized (Pedersen & Gliemann, 1981). Uptake is linear with time until approximately 3 minutes when the intracellular increase in concentration of this sugar inhibits further phosphorylation and uptake decreases (Pedersen & Gliemann, 1981). Another glucose analogue, 3-0-methylglucose, unlike 2-deoxyglucose, cannot be trapped by phosphorylation (Olefsky, 1978). The time course of uptake in adipocytes is rapid and curvilinear due to the rapid filling of the relatively small intracellular water space (Olefsky, 1978), making it imperative to measure uptake during the inital few seconds. Optimal conditions and rapid assay methods have been well described, enabling accurate determinations of glucose transport using 3-0-methylglucose (Olefsky, 1978; Ciaraldi et al., 1979; Whitesell & Gliemann, 1979; Pedersen & Gliemann, 1981; Czeh, 1976; Whitesell & Abumrad, 1986). More recently, methods have been described to facilitate determination of glucose transport in small samples of human fat cells obtained by needle biopsy (Yki-Jarvinen et al., 1986) and NMR-spin transfer procedures to study the fast chemical exchange of D-[1-^{13}C]-glucose in human erythrocytes (Kuchel et al., 1987).

Understanding the structure of the glucose transporter is well in advance of the understanding of its mechanism of action. Insulin appears to increase glucose transport in adipocytes by increasing the number of glucose transporters in the plasma membrane by a rapid and reversible translocation of transporter from intracellular pools (Karnieli et al., 1981; Wang, 1987, Matthaei et al., 1987; Wardzala et al., 1978; Karnieli et al., 1986). This is different from tissues such as the liver that do not require insulin to transport glucose and where the majority of the glucose transporters are in the plasma membrane and only a small number are seen in the micosomal fraction (Carter & Crofford, 1986). Although, several glucose transporter species have been identified intracellularly in adipocytes, only one isoform

appears to be translocated to the plasma by insulin (Matthaei et al., 1987).

Although much of the kinetic work has been done utilizing rat adipocytes, the human glucose transporter shares many characteristics with the rat adipocyte (Pedersen & Gliemann, 1981; Ciaraldi et al., 1979). However, stimulation of glucose transport by insulin in human adipocytes is relatively small compared to rats due to approximately one half the number of functional membrane transporters (Karnieli et al., 1986; Pedersen & Gliemann, 1981).

Translocation of glucose transporters by insulin to the plasma membrane is supported by evidence that insulin increases the V_{max} of glucose transport with little or no effect on the K_m (Olefsky, 1978; Wang 1987; Ciaraldi et al., 1979; Okuno & Gliemann, 1987). The increase in glucose transporters to the plasma membrane with insulin stimulation is of a reasonable magnitude to account for enhanced glucose transport with insulin stimulation (Karnieli et al., 1981a; Wardzala et al., 1978). There is, however, one report of a ten-fold decrease in K_m for transport of glucose with insulin (Whitesell & Abumrad, 1985). Recently this finding was reevaluated under similar experimental conditions and it was concluded that insulin causes a 15 to 30 fold increase in the maximum velocity for transport of glucose and a concomitant decrease in the half saturation constant, if present is insignificant (Okuno & Gliemann, 1987).

The mechanisms by which insulin stimulates glucose transport are not clear. Changes in membrane fluidity have been suggested, because transport is sensitive to temperature-induced alterations in fluidity (Ezaki & Kono, 1982). Reconstitution of the glucose transporter in phospholipid vesicles demonstrated that the transporter is sensitive to changes in the lipid bilayer (Sandra & Fyler, 1982; Melchior & Czech, 1979). Recently, it was proposed that the processes of insulin internalization and glucose transport are linked (Jochen & Berhanu, 1987). Insulin has also been reported to induce phosphorylation of a membrane protein with characteristics of the glucose transporter (Horuk et al., 1985). Glucose transport is an energy dependent process making insulin modulation of cAMP levels, by activiating phosphodiesterase, a possible critical step in eliciting the effects of the hormone on glucose transport (Lonnroth et al., 1987; Kono et al., 1977).

Glucose Utilization

In addition to glucose transport, insulin directly promotes various aspects of intracellular glucose metabolism, such as glucose oxidation and glucose incorporation into lipids in adipocytes from rats, glycogen synthesis and lactate production in muscle. Due to past difficulties incurred in measuring glucose transport, many researchers have relied on measurements of the various parameters of glucose metabolism as indirect estimates of transport activity. This is justifiable if glucose transport across the plasma membrane limits its subsequent intracellular phosphorylation and further metabolism. However, transport, at least in human (Pedersen et al., 1982c) and rat adipocytes (Czech, 1976), does not appear in most cases to be the rate-limiting step at physiological glucose concentrations for cellular glucose utilization. Intracellular measures of glucose utilization involve a complex system distal to the membrane and may be affected by factors in addition to insulin that influence substrate availability and enzyme activities. Diet composition (Salans et al., 1981), energy deprivation (Olefsky, 1976a) and disease states such as obesity (Harrison et al., 1976) and diabetes (Sinha et al., 1987; Taylor et al., 1984) have all been shown to influence insulin mediated intracellular glucose metabolism.

Fatty acid biosynthesis in adipose tissue in rats is regulated in the short term by insulin. Insulin has been demonstrated to increase both the rate of fatty acid synthesis and the activity of the rate limiting enzyme in this pathway, acetyl CoA carboxylase (Brownsey & Denton, 1982). In isolated adipocytes, insulin stimulates synthesis of the membrane phospholipids, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Casals et al., 1986; Pennington & Martin, 1985) but inhibits phospholipid methyltransferases (Merida & Mato, 1987).

Measurement of glucose utilization/disposal utilizing multiple clamp techniques, where plasma insulin and glucose levels can be manipulated and measured, have been used to assess insulin action *in vivo*. Results from these techniques have been found to correlate well to *in vitro* measurements of insulin action (Ciraldi et al., 1981). Although not a direct measure of glucose utilization, the ability of insulin to inhibit caffeine or epinephrine stimulated lipolysis

has been examined in both normal (Olefsky, 1977) and a variety of insulin resistant states (Stevens et al., 1981; Foley et al., 1986; Pedersen & Hjollund, 1983) as a measure of cellular insulin action.

Despite being influenced by many factors post-insulin binding, glucose utilization provides a useful measurement of cellular insulin action.

Relationship Between Insulin Binding and Insulin Action

Insulin resistance and decreased cellular insulin receptors are important pathophysiological features of insulin resistant states (Olefsky & Kolterman, 1981; Pedersen et al., 1982b; Taylor et al., 1984; Sinha et al., 1987). A number of studies have successfully quantitatively correlated insulin binding to receptors and insulin action (Gliemann et al., 1975; Harrison & King-Roach, 1976). Based on some of the available data on the relationship between insulin binding and glucose transport, a computer model to predict changes in glucose transport from insulin binding has been developed (Mangnall et al., 1984). However, due to the presence of spare or excess insulin receptors, the relationship between decreased number insulin receptors and insulin resistance is not straightforward (Gammeltoft & Gliemann, 1973a; Olefsky, 1976a). The concept of spare receptors results from the observation that most insulin sensitive pathways are maximally activated at hormone concentrations that occupy less than the total number of available receptors (Andersen et al., 1977; Gammeltoft & Gliemann, 1973a). Mechanistic interpretations of insulin dose response data is complicated by the fact that the proportion of spare receptors varies with cell type and is also a function of which biological action is measured. For example, in the human adipocyte the conversion of glucose to lipid was half-maximally stimulated when receptor occupancy was about 20-30% (Andersen et al., 1977), while in the rat adipocyte insulin stimulation of glucose oxidation or lipogenesis is maximally stimulated at 2% occupancy (Kono & Barham, 1971; Gammeltoft & Gliemann, 1973a).

The assumption that the amount of insulin bound to a cell is the only factor required to generate insulin action is a rather simplistic view of insulin function and neglects the fact

that the receptor is composed of two components, a binding and signal generating component. In this model, although binding is required for signal generation, binding does not guarantee signal transmission. Comparisons between insulin binding and function are more adequately assessed when the cellular action of insulin is considered in relation to the amount of insulin bound.

D. Intracellular Mediators of Insulin Action

It is well accepted that the binding of insulin to specific cell surface receptors activates certain transmembrane and intracellular signal mechanisms that couple insulin binding to insulin action. However, the specific details of these proposed molecular signals are not completely understood.

Insulin binding stimulates autophosphorylation of the insulin receptor on tyrosine residues (β-subunit) and activates an endogenous insulin receptor kinase, postulated to be critical in the mechanism of insulin action (Van Obberghen et al., 1984). Recently it was questioned if autophosphorylation of the receptor is necessary for kinase activation, because even with complete blockage of β-subunit autophosphorylation, insulin could still stimulate the endogenous kinase activity of the receptor (Morrison & Pessin, 1987). Insulin also activates several serine specific kinases that are tightly associated with the receptor but, unlike the tyrosine kinases, are not an integral part of the membrane (Van Obberghen et al., 1984). Recent studies have implicated the receptor's intrinsic tyrosine kinase activity in insulin stimulated cellular action (Ebina et al., 1987; Morgan & Roth, 1987). Futher support of a role for the insulin receptor kinase in the biologic actions of insulin come from the demonstration that agents that mimic some cellular insulin actions, such as vanadate and hydrogen peroxide, also stimulate tyrosine kinase in intact adipocytes (Kadota et al., 1987).

An association between insulin resistance and defects in the activity of receptor kinase has been reported in cultured cells (Hari & Roth, 1987), obese animals (Arner et al., 1987), streptozotocin-induced diabetes (Kadowaki et al., 1984) and individuals with noninsulin dependent diabetes (Freindenberg et al., 1987).

Alterations in phosphorylation of proteins have been demonstrated to be an important early event in the regulatory mechanisms of many metabolic pathways (Krebs & Beavo, 1979. It has been suggested that the phosphorylation of cellular substrates by the insulin receptor kinase might be the initial event in transmission of the insulin signal (Kadota et al., 1987; Jarett & Kiechle, 1984; Larner et al., 1984). Calcium-dependent, phospholipid-sensitive protein kinase (protein kinase C), a protein widely distributed in tissues (Kuno et al., 1980), has been proposed as a mediator of insulin action (Christensen et al., 1987; Cherqui et al., 1986; Witters et al., 1985). Support for this hypothesis comes from the demonstration that both *in vitro* and *in vivo* protein kinase C phosphorylates the glucose transporter (Witters et al., 1985) and agents known to activate or inhibit protein kinase C mimic or markedly decrease insulin stimulated glucose metabolism (Cheriqui et al., 1986; Christensen et al., 1987; Van De Werve et al., 1987). Recently, a defect in insulin stimulated protein kinase C was identified in insulin resistant tissues of genetically obese mice (Van De Werve, et al., 1987).

Both direct and indirect evidence supports a role for insulin in the generation of several other mediators of, at present, unknown chemical nature that mimic the effects of the hormone on a variety of intracellular enzymes. Several complex chemical mediators, that act on a variety of insulin sensitive enzymes, have been described that are produced in response to insulin in both subcellular systems and intact cells (Jarett & Kiechle, 1984; Larner et al., 1982; Haystead & Hardie, 1986). The primary function of these plasma membrane generated mediators appears to be their ability to alter the state of protein phosphorylation and by this process alter the activities of many of the regulatory enzymes of glucose metabolism (Jarett & Kiechle, 1984; Larner et al., 1982). The generation of these mediators is reduced by feeding high fat diets and the diabetic state (Begum et. al, 1982; Jarett & Kiechle, 1984).

However, the fact that not all insulin sensitive enzymes are modulated by phosphorylation-dephosphorylation mechanisms (Jarett & Kiechle, 1984; Merida & Mato, 1987) supports the existence of other insulin mediators. Recently, a novel insulin sensitive glycophospholipid was identified whose polar head group mimicked the effect of insulin on methyltransferase (Kelly et al., 1986). Insulin has also been shown to induce the rapid

hydrolysis of a membrane glycolipid that can regulate the activity of 3'5'(cyclic) adenosine monophosphate phosphodiesterase (Saltiel et al., 1986). More recently, it has been suggested that insulin hydrolyzes this novel phosphatidylinositol-glycolipid by stimulating phospholipase C and then the hydrolysis products activate protein kinase C (Saltiel et al., 1987).

Although it has been known for many years that activation of many membrane receptors stimulates phosphatidylinositol turnover, it has only been in the past few years that the physiological function of phosphatidylinosides has been appreciated (Hokins, 1985). Insulin is known to rapidly stimulate phosphatidylinositol breakdown by activation of phospholipase C (Goldman & Rybicki, 1986). Evidence exists suggesting that the immediate phosphodiesterase cleavage products inositol-triphosphate (IP_3) and diacylglycerol have a role in mediating insulin action (Goldman & Rybicki, 1986; Cheriqui et al., 1986; Farese et al., 1982). Inositol-triphosphate has been shown to be involved in insulin modulation of the pyruvate hydrogenase complex (Cheriqui et al., 1986), receptor kinase activity (Sweet et al., 1987) and glucose transport (Goldman & Rybicki, 1986) and the diacylglycerol product in activating protein kinase C (Cheriqui et al., 1986). The membrane content of phosphatidylinositol may also be important because phosphatidylinositol is an effective stimulator of insulin receptor α-subunit autophosphorylation (Sweet et al., 1987).

A role for cAMP has also been proposed in insulin mediated action because insulin, by stimulating phosphodiesterase activity, reduces cAMP levels in adipocytes (Zinman & Hollenberg, 1974; Lonnroth & Smith, 1986; Lonnroth & Smith, 1987). The ability to modulate cAMP concentration has been suggested to be a critical step for the hormone to elicit its effects on lipolysis (Lonnroth & Smith, 1986; Axelrod et al., 1986) and glucose transport (Lonnroth et al., 1987). However, other researchers report no consistent effect of insulin on cAMP levels in adipocytes (Larner et al., 1982)

As insulin induces numerous short-term and long-term anabolic effects in cells, it is unlikely that a single messenger mediates all the known cellular actions of this ligand. As knowledge increases on the function of insulin in both the normal and disease states, so does the list of possible cellular mechanisms of its action. In addition to the mediators already

discussed, it has been suggested that endogenous adenosine content (Klein et al., 1987), cytosolic free calcium levels (Draznin et al., 1987) and guanine nucleotides (G-proteins) (Pennington, 1987; Rapiejko et al., 1986) may also be involved in modulating insulin action in cells.

E. FACTORS AFFECTING INSULIN BINDING AND ACTION

Diet

Both the quantity and composition of energy provided in the diet alter insulin stimulated metabolism *in vitro* and *in vivo*.

Energy Imbalance

Acutely fasting (24 hours) rats increases insulin binding but decreases both basal and insulin mediated glucose oxidation and transport in adipocytes (Olefsky, 1976). Alterations in insulin binding to adipocytes during a short fast appear due to increases in receptor affinity, when determined by Scatchard analysis (Olefsky, 1976a; Koltermen et al., 1979a). As the length of the fast increases, there is a progressive decrease in basal and insulin stimulated glucose metabolism and an increase in adipocyte insulin binding (Olefsky, 1976a). Chronic starvation in rats (longer than 48 hours; Olefsky, 1976a) and man (longer than 7 days; Kolterman et al., 1979a; Engfeldt et al., 1985) results in an insulin resistant state of glucose metabolism in adipose tissue. Insulin binding to adipocytes during chronic starvation regimes in man has been reported to be increased (Kolterman et al., 1979a) or unchanged (Engfeldt et al., 1985). It has been suggested that the effects of energy deprivation on insulin binding and action may vary in man depending on the fat depot studied (Engfeldt et al., 1985). It is, however, quite clear that during food deprivation there appears to be little relationship between insulin binding and insulin action.

Fasting is a rather dramatic state of negative caloric balance that can only be carried out for a short period of time. Hypocaloric regimes increase insulin binding and decrease insulin mediated glucose transport in rats (Olefsky, 1976a). However, weight reduction,

induced by hypocaloric diets in obese subjects, has been shown to improve insulin action (Krotkiewski et al., 1985), suggesting that there may be inherent differences in the regulation of insulin mediated glucose metabolism associated with caloric restriction in the obese state.

It has been suggested that insulin resistance accompanying obesity results from the over consumption of energy. Inducing obesity in normal weight individuals by the chronic ingestion of a hypercaloric diet for three to four months significantly decreased insulin action in adipose tissue (Salans et al., 1974). Contrary to this, the short term ingestion for two weeks of a hypercaloric diet (160% of usual intake) by moderately obese individuals was reported to increase basal and insulin stimulated glucose transport and metabolism without changing insulin binding (Kashiwagi et al., 1985). These observations suggest that the short and long term effects of hypercaloric regimes on insulin action may be different.

Carbohydrate/ Fat Ratio

The pioneering work of Himsworth (1934) demonstrated that feeding high fat low carbohydrate diets reduces glucose tolerance and alters the effectiveness of insulin. More recently, studies in both man and animals have demonstrated that high fat low carbohydrate diets induce a state of insulin resistance to the actions of glucose transport, glucose oxidation and lipogenesis (Olefsky & Saekow, 1978; Salans et al., 1981; Lavau et al., 1979; Smith et al., 1974; Opeindipe & Bray, 1974; Maegawa et al., 1986; Susini & Lavau, 1978; Ip et al., 1977; Ip et al., 1976). High fat diets have also been shown in muscle tissue to impair insulin mediated glucose transport (Grimditch et al., 1987; Grundleger & Thenen, 1982; Susini & Lavau, 1978; Maegawas et al., 1986), glucose oxidation (Grundleger & Thenen, 1982; Susini & Lavau, 1978), lipogenesis (Susini & Lavau, 1978), glycolysis (Grundleger & Thenen, 1982) and glucose incorporation into glycogen (Grundleger & Thenen, 1982; Maegawa et al., 1986). Studies in man (Kolterman et al., 1979a) and animals (Susini & Lavau, 1978; Opeindipe & Bray, 1974) clearly demonstrate that high fat diets compared to high carbohydrate diets significantly reduce *in vivo* glucose utilization. Changing the carbohydrate/fat ratio in the diet produced rapid alterations in insulin action in two weeks or less (Ip et al., 1976; Olefsky & Saedow, 1978; Lavau et al., 1979; Maegawa et al., 1986; Grundleger & Thenen, 1982).

Although insulin resistance both *in vitro* and *in vivo*, caused by substituting dietary fat for carbohydrate, is well documented, the underlying mechanisms remain unclear. Insulin binding to adipocytes (Olefsky & Saedow, 1978; Ip et al., 1976; Kolterman et al., 1979b; Maegawa et al., 1986) and muscle (Grimditch et al., 1987; Maegawa et al., 1986; Grundleger & Thenen, 1982) has been reported to be reduced in animals fed high fat low carbohydrate diets. The magnitude of the decrease in the amount of insulin bound increases as the insulin concentration increases *in vitro* (Ip et al., 1976). Scatchard analysis suggests that alteration in insulin binding by short term (5 days) consumption of high fat diets is caused by a reduction in receptor affinity and in the long term (2 weeks or more) by reduction in the number of functional receptors (Kolterman et al., 1979b). However, not all studies agree on this point, because some studies did not change receptor binding by feeding animals high fat diets (Salans et al., 1981; Lavau et al., 1979).

Due to the design of most animal feeding studies directed at assessing the effect of alterations in carbohydrate/fat in the diet, it is impossible to determine whether or not it is the presence of fat or the absence of carbohydrate or a combination of both that influence insulin action. The carbohydrate source in the diet has been shown to alter insulin action. Keeping the fat content constant and feeding a high carbohydrate diet of simple sugars (67% of energy as sucrose) to rats, as compared to a more complex carbohydrate source, improved glucose tolerance and enhanced *in vivo* glucose uptake over a wide range of plasma insulin levels (Kergoat et al., 1987). A high carbohydrate (67% energy as glucose), fat-free diet decreased insulin binding but enhanced insulin stimulated glucose metabolism (Olefsky & Saekow, 1978). Glucose when given as a 50% solution, induced a rapid decrease in insulin binding but increased glucose metabolism (Livingston & Moxley, 1982). Therefore, it appears that in isolated rat adipocytes high glucose or sucrose diets enhance the effects of insulin on glucose metabolism. In contrast, in humans high sucrose or simple carbohydrate diets have been clearly demonstrated to impair glucose tolerance and insulin binding (Beck-Nielsen et al., 1978; Misbin, 1981). The addition of fiber to the diet is reported to improve both *in vivo* and *in vitro* insulin action in man (Hjollund et al., 1983). The protein content of the diet may

also be important as a very high protein carbohydrate-free diet significantly decreased adipocyte insulin sensitivity and response to the inhibitory effects of insulin on noradrenaline stimulated lipolysis (Kettelhut et al., 1985).

As with carbohydrate, the fat composition in dietary studies has not been adequately assessed. Most studies utilize both unphysiological levels (greater than 67% of energy) and saturated sources of fat such as lard and hydrogenated vegetable oils. Several of the studies (Olefsky & Saedow, 1978; Ip et al., 1976; Susini & Lavau, 1978) fed diets that were clearly deficient in essential fatty acids, a condition known to alter insulin binding and action (Demeyer et al., 1974). Although it has been demonstrated in both man and animals that alterations in the fatty acid composition of the diet influence the composition of adipose tissue structural and stored lipids (Carroll, 1965; Tove & Smith, 1960; Field & Clandinin, 1984; Field et al., 1985), the effect of these diet-induced alterations on insulin action has received little attention. Feeding a polyunsaturated fat (safflower oil) as compared to a saturated fat high in medium chain fatty acids (coconut oil) for four weeks at 35% of energy significantly increased insulin stimulated glucose oxidation and incorporation into lipids (Awad, 1981). Although this study clearly demonstrated an effect of diet fat, the polyunsaturated to saturated fatty acid ratio (P/S) fed was not physiological (8.0 versus 0.1) with respect to the ratio habitually consumed by man (Field et al., 1985). Feeding a high polyunsaturated fat diet (20% w/w), as compared to a highly saturated fat diet, for 8 months to weanling rats did not affect the weight of the fat pad or adipocyte cell size and number (Kirtland & Gurr, 1978). These factors have been suggested to influence cellular action of insulin (Yki-Jarvinen, 1984; Amatruda et al., 1975; Foley et al., 1986).

In the past few years, the therapeutic role of long chain polyunsaturated *w*-3 fatty acids contained in fish oils has received much attention. Recently, it was reported that replacing 6% of the *w*-6 fatty acids in safflower oil with long chain polyunsaturated *w*-3 fatty acids from fish oil prevented the development of insulin resistance associated with high fat diets (Strolien et al., 1987). This improvement in the *in vivo* action of insulin in the rat by feeding *w*-3 fatty acids may be due to the known effect of these fatty acids on membrane

composition (Garg et al., 1988) and/or eicosinoid synthesis (Samuelsson et al., 1978). Consumption of *w*-3 fatty acids may offer additional benefits; it has been recently hypothesized that enrichment of *w*-3 fatty acids in the membranes of the beta cell may, by altering substrates for membrane bound enzymes cyclooxygenase and lipooxygenase, stimulate insulin secretion (Lardinois, 1987)

It has been generally agreed that high fat diets, particularly those high in polyunsaturated fat, suppress *de novo* hepatic fatty acid synthesis (Clark et al., 1977). Early work by Romsos and Leveille (1974) in meal-fed rats suggest that as much as 70% of the *de novo* fatty acid synthesis in this animal took place in the adipose organ. Recently, it was demonstrated that in high fat-fed rats, adipose tissue can continue to be a source of *de novo* fatty acid synthesis but substituting saturated for polyunsaturated fats did not significantly alter this rate (Nelson et al., 1987). A high saturated fat diet, compared to a high carbohydrate diet, was reported to decrease the activity of some of the intracellular enzymes involved in glucose metabolism (glucose-6-phosphate and 6-phosphogluconate dehydrogenases, acetyl CoA carboxylase, pyruvate dehydrogenase and malic enzyme) in adipocytes (Begum et al., 1982; Lavau et al., 1979).

Although the mechanisms responsible for insulin resistance induced by high fat diets remain unclear, several additonal factors associated with this nutritional manipulation have been implicated. High carbohydrate diets are reported to increase plasma insulin levels in many (Ip et al., 1976; Salans et al., 1981; Kolterman et al., 1979b) but not all studies (Olefsky & Saekow, 1978). However, the lower plasma insulin levels observed by feeding high fat low carbohydrate diets does not produce the classical 'upregulation' of insulin receptor binding (Kolterman et al., 1979b). It has been suggested by some that high carbohydrate diets increase hormone degradation (Olefsky & Saekow, 1978), but others report no effect (Ip et al., 1976). Coupling between the insulin receptor and glucose transport does not appear to be altered, as the amount of insulin bound per glucose transported was unaffected by diet (Olefsky & Saekow, 1978; Grundleger & Thenen, 1982). Insulin resistance to glucose transport by high fat/low carbohydrate diets may be a consequence of a decreased number of

glucose transporters on the adipocyte plasma membrane (Salans et al., 1981) caused by depletion of intracellular transporters (Hissin et al., 1982). The increased body weight (Grundleger & Thenen, 1982; Maegawa et al., 1986) and adipocyte cell size (Smith et al., 1974) reported by feeding low carbohydrate/fat ratio diets has led to the suggestion that high fat diets produce an insulin resistant state similar to that seen in the obese state. However, significant alterations in adipocyte insulin action can be produced by isocaloric substitution of fat for carbohydrates in a time period where no difference in body weight or cell size were observed (Ip et al., 1976; Salans et al., 1981; Ogeindipe & Bray, 1974).

Most of the human work examining the effect of diet on insulin action has been conducted in individuals with diabetes. It has been shown that reducing the fat content and increasing the P/S ratio, the complex carbohydrate and fiber content of the diet improves both *in vivo* and *in vitro* insulin action in both noninsulin dependent (Hjollund et al., 1983; Ward et al., 1982) and insulin dependent (Pedersen et al., 1982b) diabetic patients.

The energy providing nutrients in the diet have a major effect on overall insulin action. However, due to the use of unphysiological diets with respect to the carbohydrate and fat sources in animal studies, application to the human is difficult. Animal studies suggest that high saturated fat diets are associated with both *in vitro* and *in vivo* insulin resistance. From available clinical studies, it appears that replacing saturated fats in the diet with polyunsaturated fats may improve cellular insulin action.

Membrane Lipid Composition

Current understanding of the organization of biological membranes relies on the fluid mosaic model proposed by Singer and Nicholson (1972) in which proteins are embedded to varying degrees in a lipid bilayer. In morphological studies, the insulin receptor (Jarett & Smith, 1974) and glucose transporter (Matthaei et al., 1987) are located on the cell surface, acting within the lipid matrix of the plasma membrane. Because of the intimate contact of the receptor and glucose transporter with membrane lipids, it is conceivable that alterations in the composition or physical state of adjacent phospholipids could influence cellular insulin action.

The function of other membrane associated proteins (Clandinin et al., 1985), including hormone mediated functions (Neelands & Clandinin, 1983), has been clearly demonstrated in a variety of tissues to be sensitive to alterations in membrane lipid composition.

A number of studies have indirectly suggested that the physical state of the plasma membrane can influence membrane associated insulin binding and glucose transport (De Meyts et al., 1978; Amatruda & Finch, 1979; Melchoir & Czech, 1979). Early studies demonstrated that insulin binding and action can be altered by subjecting the plasma membrane to various treatments that disrupt the protein and lipid components of the membrane. For example, treatment of cells with trypsin severely reduces insulin binding and insulin stimulated glucose oxidation (Cuatrecasas, 1971). Phospholipase-induced hydrolysis or displacement of membrane lipids alters insulin binding and reduces hormone-mediated response (Rodbell, 1966). An increase in temperature is associated with an increase in number of available insulin binding sites (De Meyts et al., 1978; Amatruda & Finch, 1979) and enhancement of insulin stimulated glucose transport (Melchoir & Czech, 1979; Amatruda & Finch, 1979). These temperature-dependent alterations in insulin binding and action have been interpreted to result from changes in membrane fluidity (Amatruda & Finch, 1979). Recently, a significant inverse relationship was reported between the level of insulin binding and membrane fluidity in offspring from diabetic pregnancies (Neufeld & Corbo, 1986). The concept of membrane fluidity refers more specifically to properties of the hydrophobic core of the membrane. These properties depend not only on the temperature but also the chemical composition of the membrane, mainly its cholesterol and phospholipid content and the length and degree of unsaturation of the fatty acyl chains of the phospholipid core.

Supplementation of culture medium with specific lipids is a rapid and widely used method for significantly modifying membrane lipid composition and studying the effect on membrane proteins (Spector et al., 1979; Williams et al., 1974). Studies done by culturing several cell lines in media of different fatty acids suggest that the membrane lipid composition may have a profound influence on both the binding properties of the insulin receptor and the cellular action of insulin. Increasing the polyunsaturated fatty acid composition and

membrane fluidity of Friend erythroleukemia cells, by growing in media enriched in polyunsaturated fatty acids, as compared to those enriched in monounsaturated fatty acids, significantly increased insulin binding (Ginsberg et al., 1981). From Scatchard analysis, the increase in binding was due to an increased number of available binding sites (Ginsberg et al., 1981). A saturated media, as compared to a monounsaturated media, decreased both the affinity and number of insulin receptors and impaired insulin mediated glucose transport in 3T3-L1 preadipocytes (Grundfeld et al., 1981). However, not all cell lines respond similarly to alterations in membrane fatty acid composition. Rat hepatoma cells (Bruneau et al., 1987a) and 3T3-L1 preadipocytes (Henson et al., 1981), grown in culture medium enriched in linoleic acid, demonstrated decreased insulin binding. Inducing extensive fatty acyl changes in the phospholipids of bovine endothelial cells did not alter the binding or the cellular processing of insulin (Bar et al., 1984). Resistance to insulin mediated amino acid transport and glycogen synthesis were produced in the hepatoma cell line (Bruneau et al., 1987b) despite an increased membrane polyunsaturated fatty acid content and the corresponding increase in fluidity.

Another approach to studying the role of membrane lipids on the properties and function of the insulin receptor *in vitro* is the use of reconstruction techniques. These techniques permit alterations in specific membrane phospholipids via liposome fusion and lipid substitution or by reconstituting purified or partially purified systems into bilayers of specific lipid compositions. Reconstitution of solubilized erythrocyte insulin receptors into highly unsaturated phospholipid vesicles, compared to saturated vesicles, increased the number of available binding sites but reduced the affinity of the receptor for insulin (Gould et al., 1982). These alterations in binding were produced without modifying vesicle size or the orientation of the receptor within the bilayer (Gould et al., 1982). The glucose transport system of the adipocyte plasma membrane has also been shown to be sensitive to specific alterations in both the polar head group (Sandra & Fyler, 1982; Sandra et al., 1984) and acyl chain composition (Pilch et al., 1980) of the surrounding membrane lipids. It was demonstrated by reconstitution of the adipocyte D-glucose transporter into phospholipid bilayers of varying fluidity that optimal transport requires a fully fluid bilayer and becomes

inoperative when placed in a crystalline bilayer (Melchior & Czech, 1979).

It has been demonstrated in a variety of tissues, including adipose tissue (Field et al., 1985), that the nature of fat consumed can alter membrane phospholipid compostion and that these diet-induced alterations in membrane lipids influence many membrane-associated functions (Clandinin et al., 1985; Neelands & Clandinin, 1983; Garg et al., 1988; Hargreaves & Clandinin, 1987). There is limited evidence that physiologically diet-induced alterations in membrane composition can change insulin receptor binding and action. In this regard, the *in vitro* work in cell cultures was extended to the intact animal by growing Ehrlich ascites cells in mice fed diets containing a high level of polyunsaturated or monounsaturated fat (Ginsberg et al., 1982). The highly polyunsaturated membranes from the tumour cells grown in mice fed the polyunsaturated fat demonstrated enhanced insulin binding due to an increased number of receptors (Ginsberg et al., 1982).

The time required to alter membrane composition by diet modifications relies to some extent on the turnover of membrane lipids. The turnover of stored triglycerides in adipose tissues has been estimated to be approximately one year (Hirsh, 1960). Although there is no data directly assessing plasma membrane turnover in the adipocyte, one can assume that it is much shorter. In growing animals, significant diet induced alterations in the composition and function of mitochondrial (Innis & Clandinin, 1981) and liver plasma (Neelands & Clandinin, 1983; Morson & Clandinin, 1986) membranes were produced after only two to three weeks of diet treatment.

Molecular mechanism(s) by which membrane alterations influence insulin binding and action are not clear. A few hypotheses have been suggested. Gould et al. (1979) proposed that the accessibility of the insulin receptor for its ligand may be controlled by conformational changes in the receptor that require a more fluid environment and cannot take place when the phospholipid fatty acyl chains surrounding the receptor are saturated. Mobility within the lipid bilayer may also be an important factor in insulin action. When reconstituted into phospholipid vesicles, insulin-stimulated glucose transporter activity increased with increasing membrane fluidity (Melchoir & Czech, 1979). Insulin stimulation, as determined by lateral

diffusion of lipid probes, was demonstrated to alter lipid dynamics of hepatic plasma membranes (Stuschke & Bojar, 1985). However, the relative response of fluorescent and spin probes suggests that this effect of insulin on membrane lipid ordering may be confined to specific domains within the membrane matrix (Hyslop et al., 1984). Ginsberg et al. (1982) suggested that the effects of lipid modification of membranes may be due to alterations in the quaternary structure of the receptor. These researchers suggested that the receptor may exist in two states, monomeric state of high binding capacity, low ligand affinity, and a polymeric state of low capacity, high affinity and that the level of membrane saturation may determine which state is favoured (Ginsberg et al., 1982).

Alterations in insulin action could also be mediated by changes in the synthesis or turnover of specific membrane lipids. Insulin treatment of isolated rat adipocytes has been demonstrated to induce an acute increase in membrane phosphatidylinsoitol levels (Farese et al., 1982). The activity of enzymes involved in *de novo* synthesis (CDP-diglycerol inositol transferase) and degradation (phospholipase C) of phosphatidylinositiol are reported to be sensitive to both polyunsaturated fatty acids (Takenawa & Nagai, 1982) and to the lipid composition of the membrane they are located in (Irvine, 1982). Modification of the lipids already present in the membrane may be important, as systematic alterations in the content and composition of membrane phospholipids *in vitro* have been shown to influence insulin binding and action (Sandra et al., 1984). Lastly, it is reasonable to assume that membrane alterations may modify the transduction of the signal generated in the plasma membrane by hormone binding, thereby altering intracellular insulin action.

Although liposome reconstitution and cell culture techniques are attractive approaches to study the role of membrane lipids on cellular insulin function, several problems arise in the interpretation of results. The possibility that liposome vesicles influence insulin binding and action by a process independent of membrane alterations can not always be ruled out. In cell culture work most of the cellular lipids, including membrane phospholipids, are derived from the material that is taken up from the medium making both the composition and form of the lipid supplement critical if an *in vivo* interpretation is attempted. In addition, most insulin

sensitive cells are difficult to culture, as mutations are rapidly introduced and many cellular functions lost with time. Thus, much of the work has been done utilizing tumour cell lines. Very large and likely unphysiological alterations are induced in membrane lipids, again making physiological interpretation difficult, if not impossible. Reporting alterations in total cellular membrane phospholipid, may not reflect the changes in structure which are most closely related to change in function and neglects the observation that in other tissues, alterations in the composition and content of specific membrane phospholipids may be related to changes in function (Neelands & Clandinin, 1983; Clandinin et al., 1985).

The currently available methods for measuring membrane fluidity employed in many studies are limited to measuring overall membrane fluidity and may not be representative of or sensitive to localized changes in the microenvironment surrounding the insulin receptor or glucose transporter. In a recent study, grossly disordering the membrane by benzyl alcohol treatment significantly reduced both insulin binding and glucose transport but did not change the amount of glucose transported per insulin bound (Hyslop et al., 1987). The authors suggested that the receptor and glucose transporter may be located within a structurally distinct region of the membrane not influenced by disordering of the membrane lipid matrix by this compound (Hyslop et al., 1987).

It has been clearly demonstrated that insulin binding and glucose transport are sensitive *in vitro* to manipulation of membrane physical and chemical properties. Although demonstrated for some other membrane mediated functions, there is limited data on the relationship of diet to membrane compostion and the effect of membrane alterations on cellular insulin function. Furthermore, only a few studies have measured concomitant changes in insulin action with changes in binding. Moreover, an insulin resistant state (i.e. diabetes) is associated with specific alterations in membrane fatty acid composition (Faas & Carter, 1980; Worchester et al., 1979) and these may play a role in the etiology of insulin resistance (Sandra & Fyler, 1982). If change in dietary fat can be shown to manipulate insulin action, via membrane alterations, it may offer a viable treatment to improve insulin sensitivity in hormone resistant states.

Other

Age

The reduced glucose tolerance associated with age (Pagano et al., 1981) has been suggested to be caused by age-induced alterations in insulin sensitivity, because insulin binding to adipocytes from both rats (Olefsky & Reaven, 1975) and man (Paganoet al., 1981) is reported to decrease with age. Further support for age effects on insulin action is inferred from studies where adipocytes from older animals were found to be less sensitive to the antilipolytic effects of insulin than younger animals, and this decrease in sensitivity was consistent with a reduced number of cellular receptors (Olefsky, 1977). Binding to rat adipocytes has been demonstrated to decrease with age until a critical body weight/age was reached, after which there was no further decrease in binding (Olefsky & Reaven, 1975). Difficulties exist in the interpretation of age-induced alterations in insulin binding and action because most studies compare larger fat cells from older obese animals or humans with smaller fat cells from young lean animals or humans. However, age can not be entirely ruled out as a factor influencing insulin action; when cell size is kept constant, insulin stimulated incorporation of glucose into adipocyte lipids significantly decreases with age (Taniguchi, 1986).

Gender

Although gender differences exist in the distribution of body fat, the role of gender in insulin action is not clear. Fat cells isolated from female subjects were found to be larger and when expressed per cell, demonstrated increased binding and enhanced insulin stimulated glucose transport and metabolism (Pedersen et al., 1982c). However, differences in insulin binding and glucose transport reported in this study disappeared when expressed per cell surface area (Pedersen et al., 1982c). In rats, when cell size was controlled, adipocytes from females bound more insulin, due to enhanced receptor affinity, and incorporated more glucose into lipid (Guerre-Millo et al., 1985a). Studies may be further complicated by the degree of adiposity as insulin mediated glucose disposal has been shown to be inversely related to adiposity in both normal weight males and females (Yki-Jarvinen, 1984). The role of gender

at present remains speculative but factors associated with gender, such as specific sex hormones (Tsbibris et al., 1980), influence insulin binding and action.

Tissue and Anatomical Site

Accessibility of blood cells has frequently led to use of the erythrocyte to assess insulin receptor binding. However, blood cells respond only minimally to insulin and have not been shown to have a defined role in glucose homeostatsis. In addition, the relationship between insulin binding to various blood cells populations and adipocytes remains controversial (Mandarino et al., 1984a; Pedersen et al., 1982; Olefsky, 1976). Due to the functional differences between tissues, it is not unexpected that tissue-specific differences in receptor binding exist. Therefore, it appears most logical to study insulin receptor binding in an insulin target tissue.

In regard to glucose metabolism, muscle represents the major insulin dependent organ. However, at present difficulties exist in collecting, isolating, purifying and measuring insulin action in muscle. New techniques have recently been described that may make muscle preparations more practical in the future (Arner et al., 1987; Maegawa, 1986). At present however, adipose tissue represents the most useful tissue, because it is easily accessible and yields a pure cell preparation that is extremely sensitive *in vitro* to physiological concentrations of insulin. Isolated adipocytes are more responsive to insulin than intact tissue. The direct contact of free fat cells with substances present in the bathing media has been suggested to be more similar to the *in vivo* supply of nutrients through capillaries than to the long diffusion path through tissue segments (Gleimann, 1968).

Regional differences in insulin binding and action have been reported between the two largest fat depots, subcutaneous and omental. Subcutaneous adipocytes are larger (Bolinder et al., 1983), bind more insulin (Livingston et al., 1984) and in man are more sensitive to insulin (Bolinder et al., 1983). However, in rats, subcutaneous adipocytes, as compared to other sites, are reported to be less sensitive to insulin (Guerre-Millo et al., 1985a). Although fat tissue may be metabolically heterogeneous with respect to the degree of insulin sensitivity, it is unlikely that the direction of the response is different between sites.

Plasma Insulin

Numerous studies have demonstrated that insulin can inversely regulate the number of cellular insulin receptors in a dose-dependent manner (Marshall & Olefsky, 1980; Livingston et al., 1978; Olefsky et al., 1982). This phenomenon is known as insulin-induced receptor down-regulation or up-regulation, depending on insulin levels studied. *In vitro*, the loss of receptors by insulin incubation leads to the predicted decrease in insulin sensitivity (Marshall & Olefsky, 1980; Livingston et al., 1978). However, prolonged insulin incubation is associated with effects at multiple postreceptor sites (Garvey et al., 1986; Garvey et al., 1985; Marshall & Olefsky, 1980).

Although it was suggested at one time that the rapid down regulation of insulin receptors *in vitro* may be an artifact of the incubation media (Rennie & Gliemann, 1981) there is now considerable *in vivo* evidence to support a role of plasma insulin in regulating insulin binding and action. For example, chronically injecting rats with large amounts of insulin resulted in hyperinsulinemia and a reduced number of functional insulin receptors (Kobayashi & Olefsky, 1978; Whittaker et al., 1979) that rapidly returned to normal upon withdrawal of insulin (Kobayashi & Olefsky, 1978). Similarly, in man, administration of insulin alters insulin binding (Smith, 1980) and action (Mandarino, 1984). Neither the mechanism of receptor down-regulation nor fate of lost receptors is known. It has been suggested that chronic insulin exposure may: induce the transfer of cell surface receptors to intracellular regions (Olefsky et al., 1982); cause structural or functional changes in the receptor (Garvey et al., 1986); decrease the cell's ability to degrade insulin (Marshall & Olefsky, 1980); or affect the promulgation of the insulin signal (Garvey et al., 1986). The process of insulin mediated receptor loss also appears to be an energy-requiring process (Olfesky et al., 1982).

In individuals who may have been treated for many years, the chronic hyperinsulinemia that accompanies some insulin resistant states, such as obesity (Bar et al., 1976), noninsulin dependent diabetes (Truglia et al., 1985) and insulin dependent diabetes (Pedersen et al., 1982a), have been identified as a possible factor mediating reductions in

insulin binding and action. Although infusion of physiological levels of insulin to healthy volunteers induces insulin resistance (Mandarino et al., 1984a), the lack of consistency between changes in hormone concentration and receptor binding in insulin resisistant states suggests that the role of hyperinsulinemia in the induction of insulin resistance in these disorders is likely minor.

Plasma Glucose

In vitro there is limited information to suggest an effect of glucose concentration on insulin receptor binding. However, the ambient glucose level may play a role in determining both insulin sensitivity and maximal insulin responsiveness, as both insulin sensitivity and insulin responsiveness of adipocyte lipogenesis have been demonstrated to be dependent on *in vitro* glucose concentration (Pedersen et al., 1982c). In these experiments, sensitivity to insulin increased but the maximum response decreased with increasing glucose concentrations (Pedersen et al., 1982c). Both *in vitro* and *in vivo* ability of insulin to inhibit lipolysis is reported to directly relate to glucose concentration (Arner et al., 1983; Arner et al., 1983b). Although support exists *in vitro* and in the normal state, the role of plasma glucose concentration remains to be determined in insulin resistant states, such as diabetes and obesity, that are accompanied by hyperglycemia.

Exercise

Adipocytes from trained as compared to sedentary animals have been reported to bind more insulin (Craig et al., 1981) and exhibit increased insulin stimulated glucose metabolism (Craig et al., 1981; Wardzala et al., 1980; Craig & Foley, 1984). Although cell size clearly decreases with exercise (Craig et al., 1981; Craig & Foley, 1984), the amount of glucose transported with insulin stimulation remains elevated in exercised animals even when cell size is controlled (Craig et al., 1981).

Pregnancy

Pregnancy represents a unique state of insulin resistance in man as insulin action returns to normal or near normal shortly after delivery. Attempts, both *in vitro* (Hjollund et al., 1986) and *in vivo* (Ryan et al., 1985), to determine if changes in insulin receptor function

contribute to the decreased insulin action of late pregnancy have yielded conflicting results. It has been suggested that both the tissue used to measure binding (Hjollund et al., 1986) and the time during the menstrual cycle when control subjects were examined (Bertoli et al., 1980) influence binding comparisons. During late pregnancy insulin receptor binding, as compared to normal nonpregnant women, was reported to be unchanged in blood cells (Ryan et al., 1985; Tsbibris et al., 1980). However, despite no change in insulin binding to monocytes and erythrocytes, insulin binding to adipocytes was significantly reduced (Hjollund et al., 1986).

Gestational onset diabetes, usually occurring late in pregnancy, produces an even more marked resistance to insulin mediated glucose uptake than normal pregnancy (Ryan et al., 1985). However, there is evidence that good glycemic control may prevent this further deterioration in insulin action during diabetic pregnancy (Hjollund et al., 1986). Offspring from diabetic pregnancies have been reported to demonstrate abnormal insulin binding; it was recently reported that animals born to diabetic animals bind more insulin, which was associated with an increased number of available insulin receptors (Neufeld & Corbo, 1986).

Hormones

Several of the counter-regulatory hormones including epinephrine (Cigolini et al., 1986; Baron et al., 1987), cortisol (Cigolini & Smith, 1979) and growth hormone (Bratusch-Marrain et al., 1982) impair insulin mediated glucose metabolism and have thus been implicated in the deterioration of glucose tolerance under certain metabolic conditions. Diabetes mellitus has been described as a bihormonal disorder where both insulin deficiency and absolute or relative glucagon excess contribute to the metabolic disturbances (Unger & Orci, 1975). Hyperglucagonemia may also contribute to insulin resistance; glucagon infusions were shown to impair insulin mediated glucose metabolism in normal subjects (Del Prato et al., 1987).

Although not a hormone, adenosine has been implicated as an important endogenous regulator of adipose tissue metabolism. Physiological concentrations of adenosine are reported to increase insulin stimulated glucose transport and oxidation (Green, 1983).

F. INSULIN RESISTANT STATES

Several disorders in man and animals exhibit insulin resistance. Insulin resistance is defined as a state in which a given concentration of insulin produces a subnormal biologic response.

Obesity

Obesity is the most common insulin resistant state in man, is characterized by increased concentration of circulating insulin in both the basal state and after various stimulants of insulin secretion, and is resistant to both exogenous and endogenous insulin (Rabinowitz, 1970; Olefsky et al., 1982). A large amount of work has focused on identifying the underlying defect(s) involved in the insulin resistance accompanying the obese state.

Impaired insulin binding to its receptor has been reported in monocytes (De Pirro et al., 1980; Wigand & Blackard, 1979; Bar et al.,1976; Archer et al., 1975), leukocytes (Beck-Nielsen et al., 1976), and adipocytes (Pedersen et al., 1982d; Harrison et al., 1976) obtained from obese individuals. Most of these studies identify reduction in the total number of functional cellular receptors, rather than alterations in affinity of the receptor for insulin, as contributing to decreased hormone binding (Archer et al., 1975; Amatruda et al., 1975; Beck-Nielsen et al., 1976). Attempts to correlate reductions in binding with degree of obesity (Yki-Jarvinen, 1984) and altered hormone degradation (Archer et al., 1975; Olefsky & Reaven, 1975; Amatruda et al., 1975) have met with limited success.

Although studies *in vitro* indicate that high insulin levels may induce insulin resistance at the receptor as well as the postreceptor sites (Marshall & Olefsky, 1980; Garvey et al., 1986), it is uncertain if the hyperinsulinemia accompanying obesity contributes to impaired insulin action or if it is merely a consequence of insulin resistance (Marshall & Olefsky, 1980; Livingston et al., 1978). Fasting plasma insulin levels have been clearly shown in many (Wigand & Blackard, 1979; Bar et al., 1976; Beck-Nielsen, 1976) but not all (Archer et al., 1975) studies to negatively relate to insulin binding to blood cells. Reducing the hyperinsulinemic condition of obese individuals by weight loss after consumption of

hypocaloric diets improves or corrects insulin binding to these cell populations (Archer et al., 1975; Bar et al. 1976; Beck-Nielsen et al., 1976). Insulin binding to adipocytes, however, does not appear as sensitive to circulating insulin levels as insulin binding to blood cells (Mandarion et al., 1984; Harrison et al., 1976), but adipocyte binding was reported in one study to improve with reductions in plasma insulin induced by weight loss (Pedersen et al., 1982d). The presence of an adipocyte insulin binding defect in the obese state, despite cellular resistance to insulin action, has not always been demonstrated (Arner et al., 1987).

Because the obesity is associated with an enlarged adipocyte, a decrease in receptor density on the plasma membrane is a feature often associated with the obese state (Amatruda et al., 1975; Livingston et al., 1984; Harrison et al., 1976). Changes in receptor density may help explain improvements in insulin binding associated with weight loss and reductions in cell size (Pedersen et al., 1982d).

To further examine the possibility of receptor alterations in adipocytes, various genetic (Soll et al., 1975; Vicarro et al., 1987; Freychet et al., 1972) and acquired (Soll et al., 1975;) forms of obesity have been studied in rodents. Obesity in the hyperglycemic, hyperinsulinemic ob/ob mouse is accompanied by impaired insulin binding to varous tissues including fat cells (Soll et al., 1975; Freychet et al., 1972) and muscles cells (Vicarro et al., 1987). In this model, insulin resistance is associated with a decreased concentration of receptors that can be restored to normal by therapy such as acute fasting (Soll et al., 1975) that is aimed at reducing plasma insulin levels. However, severe food restriction for 15 days decreased plasma insulin levels but the binding capacity of the adipocyte plasma membrane remained well below that of membranes from nonobese mice, suggesting that hyperinsulinemia may not be the cause of insulin resistance in this disorder (Freychet et al., 1972). A common studied rodent model of obesity has been the aged rat Wister in which there is a progressive decrease in the ability of adipocytes to bind insulin as animals get older and fatter (Olefsky & Reaven, 1975; Olefsky, 1976a). However, there are reports of increased binding to adipocytes with increased cell size and expanding cell surface (Suzuki et al., 1985; Cushman et al., 1981). In these studies, large cells retained the capacity to bind insulin in excess of that required to produce

the maximum response to insulin (Suzuki et al., 1985; Cushman et al., 1981).

Although there are reports directly relating the number of receptors on fat cells to insulin sensitivity *in vitro* (Harrison et al., 1976) and *in vivo* (Ciaraldi et al., 1981), the degree of insulin resistance reported in obesity is generally much greater than would be predicted from decreased insulin binding. Various researchers have with some success related *in vivo* insulin action using euglycemic clamps and infused insulin, or glucose tolerance tests to factors associated with obesity such as body fat (Bogardus et al, 1985), hyperinsulinemia (Olefsky, 1981), indices of the level of physical fitness (Bogardus et al., 1985) and cellular glucose metabolism (Foley et al., 1986). However, much of the work characterizing and identifying underlying mechanisms of obesity that associate insulin resistance with the obese state has been done *in vitro*, relying heavily upon the isolated adipocyte as the cell model.

Insulin stimulated adipocyte glucose transport, an early step of cellular glucose metabolism, is reported to be reduced in both human obesity (Pedersen et al., 1982d; Foley et al., 1986; Ciaraldi et al., 1981) and the aged obese rat model (Kahn & Cushman, 1985). Noninsulin dependent basal glucose transport, however, does not appear to be altered by the obese state (Pedersen et al., 1982d; Kahn & Cushman, 1985), but may depend more on plasma glucose concentration (Livingston et al., 1984). Although the relationship between the size of the adipocyte and insulin mediated glucose transport has been the subject of many studies, the results of these studies are not always in agreement. There are reports that indicate as the cell enlarges it loses ability to transport and metabolize glucose (Craig et al., 1984; Foley et al., 1986) while reports utilizing a nonobese model suggest glucose uptake increases with cell size (Hood et alo., 1984). These results implicate that something inherent to the obese state other than simply cell enlagement may alter cellular insulin action. Although the coupling between the glucose transporter and the insulin receptor appears intact in cells from obese animals (Olefsky, 1976b), differences may exist in how the cell responds to insulin (Kahn & Cushman, 1985). A depletion of glucose transporters in low-density microsomes in the basal state and a reduction in the number translocated with insulin stimulation (Kahn & Cushman, 1985) were reported in obese animals. In yet another study, obesity was again

associated with a depletion of total glucose transporters, but also associated with a reduction in their intrinsic activity at the plasma membrane (Karnieli et al., 1986a). Defects in adipocyte glucose transport, however, have not been identified in all obese animal models (Guerre-Millo et al., 1985). For example, insulin was shown to increase glucose transport in adipocytes from the obese Zucker rat to a greater extent than cells from lean littermates (Guerre-Millo et al., 1985b). This increase was attributed to an increase in the number of intracellular and plasma membrane glucose transporters (Kahn & Cushman, 1985; Guerre-Millo et al., 1985b).

Reduction in insulin stimulated intracellular glucose oxidation is reported in obese man (Salans et al., 1974; Harrison et al., 1976; Pedersen et al., 1982d) and animal models of obesity (Craig & Foley, 1984; Olefsky, 1976b; Stevens et al., 1981). As with glucose transport, cell enlargement may to some extent influence a cell's ability to metabolize glucose (Craig & Foley, 1984; Hood & Thorton, 1980; Davidson, 1975), but does not appear to influence basal (noninsulin mediated) glucose oxidation (Olefsky, 1976b). However, within an individual, cell size was found to negatively correlate with basal glucose oxidation (Harrison & King-Roach, 1976). Glucose incorporation into adipocyte lipids is reported to be reduced in the obese state (Pedersen et al., 1982d; Salans et al., 1984). The ability of insulin to inhibit lipolysis in the adipocyte is reported to be both reduced by obesity (Stevens et al., 1981; Olefsky, 1976b; Pedersen, 1982d) or unchanged (Foley et al., 1986), suggesting that the obese state does not influence all insulin mediated actions similarily.

Insulin resistance at the target cell may be caused by events postreceptor binding. For this reason, alterations in the stimulation of receptor-associated kinase activity, one of the earliest recognized events after insulin binds to the receptor (Kahn, 1985), has been examined but the findings are inconclusive. Decreases in insulin stimulated tyrosine kinase activity were reported in the heart and hepatocytes of genetically obese rats (Van De Werve et al., 1987) but were found to be normal in skeletal muscle of obese mice (Vicarro et al., 1987). Recently, a 40% decrease in insulin kinase activity was reported in skeletal muscle biopsies from obese individuals (Arner et al., 1987a), but insulin stimulated kinase activity was found in another

study to be comparable in adipocytes from control and obese subjects (Friedenberg et al., 1987).

Despite the amount of research directed at identifying and characterizing the cellular defect responsible for the *in vivo* insulin resistance associated with the obese state, many questions still remain. An alteration in insulin binding is not a universal finding and when reported is likely not large enough to explain alterations in cellular function. A decrease in intracellular function due to increased cell size is a simplistic explanation, because fasting (Pedersen et al., 1982d) reduces cell size but does not always correct the cellular defects. The macronutrient content of the diet, shown to alter insulin action (Olefsky & Saedow, 1978; Beck-Nielsen et al., 1976; Awad, 1981), is commonly overlooked when studying obesity, a disorder where diet is a major component of treatment.

Insulin Dependent Diabetes Mellitus

Most individuals with insulin-dependent diabetes require more than 20-25 units of insulin/day, the estimated mean amount of insulin secreted in the normal state to achieve glucose control (Eaton et al., 1980). The observation that glucose control is usually suboptimal suggests that in this form of diabetes there is insulin resistance. Absolute insulin deficiency accompanied by ketoacidosis induces insulin resistance in both animals (Cuthbert & Alberti, 1978) and man (Ginsberg, 1977), but it is not clear at present whether insulin replacement can restore normal insulin sensitivity.

Glucose-insulin clamp techniques have been employed to assess *in vivo* glucose metabolism in diabetic individuals. When studied at euglycemic levels, insulin-mediated glucose metabolism was significantly reduced in noninsulin dependent patients (DeFronzo et al., 1982; Del Prato et al., 1983). However, in these studies, insulin-mediated glucose metabolism/disposal was at fasting hyperglycemia levels reduced (DeFronzo et al., 1982) or unchanged (Del Prato et al., 1983) in insulin dependent diabetic patients. By employing multiple euglycemic insulin clamps it was demonstrated that, at physiological insulin concentrations, insulin dependent diabetic patients were resistant to insulin action, compared

to control subjects (Pernet et al., 1984). In contrast, at supraphysiological levels of insulin, glucose disposal in diabetic subjects was similar to controls (Pernet et al., 1984), suggesting that the abnormal tissue response to insulin in this form of diabetes may be restricted to a relatively narrow range of insulin concentrations.

Many of the studies assessing insulin resistance in diabetes fail to account for the degree of metabolic control. Insulin resistance may be secondary to the deranged metabolic state; when recently diagnosed diabetics were assessed using insulin-clamp techniques, 75% demonstrated normal insulin-mediated glucose metabolism (Ginsberg, 1977). Contrary to these findings, when HLA-identical siblings from insulin dependent diabetic subjects were compared to matched controls, despite normal fasting glucose levels, insulin sensitivity was significantly reduced in the HLA-identical siblings (Raghu et al., 1985), suggesting that impaired insulin sensitivity may be an early consequence of the disorder. Thus, the role of metabolic control in insulin action remains unclear. Using a multiple euglycemic insulin clamp, the dose response for insulin mediated glucose disposal was normal in well controlled diabetic patients but markedly reduced in diabetic patients, who required large insulin doses and routinely exhibited marked fluctuations in plasma glucose levels (Revers et al., 1984). The importance of good diabetic conrol in normalizing insulin mediated action is controversial. Strict insulin therapy with continuous subcutaneous insulin infusions did not improve insulin mediated action despite six months of near normalization of plasma glucose levels (Del Prato et al., 1983). However, in another study, six weeks of continuous subcutaneous insulin infusions to improve metabolic control, resulted in an enhanced sensitivity to insulin and a reduction in basal hepatic glucose production (Yki-Jarvinen & Koivisto, 1984).

Increases (Pedersen et al., 1983), decreases (Pedersen et al., 1982a; Pedersen et al., 1981; Pedersen et al., 1982b; Eaton et al., 1984) and no differences (Pedersen & Hjollund, 1983; Pedersen et al., 1978; Pav et al., 1985) have been reported for insulin binding to various blood cell populations from insulin dependent diabetic individuals compared to nondiabetics. This apparent discrepancy in the literature may relate to both the state of diabetic control and possible regulatory effects of ambient insulin concentration. Therapy aimed at improving

blood glucose control and preventing large fluctuations in plasma insulin levels (Pedersen et al., 1981; Pedersen et al., 1982b) normalized insulin binding to blood cells. However, the use of blood cells to assess insulin receptor binding remains questionable; reduced insulin binding to adipocytes (Pedersen & Hjollund, 1983; Smith, 1980) and decreases in insulin sensitivity, as assessed by insulin clamp techniques (Del Prato et al., 1983), have been reported in diabetic patients who exhibited normal insulin binding to erythrocytes or monocytes.

Reductions in insulin binding to fat cells from diabetic individuals have been attributed to changes in both the number of functional receptors (Pedersen & Hjollund, 1983) and the affinity of the receptor for insulin (Taylor et al., 1984). Insulin mediated cellular functions in adipocytes of glucose transport, glucose oxidation, lipogenesis and inhibition of lipolysis have been reported to be altered by the diabetic state (Pedersen & Hjollund, 1983; Taylor et al., 1984). It has been suggested that impaired insulin binding may be responsible for alterations in adipocyte glucose transport and antilipolysis and that postreceptor alterations may be the primary cause of the inability of the adipocyte to oxidize glucose and synthesize lipid (Pedersen & Hjollund, 1983).

Although impaired insulin sensitivity, determined by *in vivo* techniques, appears to be a common feature of insulin dependent diabetes, limited work has been done to characterize the cellular alterations involved in causing this resistance. The possibility that lipid alteration may be involved is suggested by the finding of lower levels of total polyunsaturated (w-6 and w-3) fatty acids in erythrocytes from poorly controlled diabetic patients (Van Doormaal et al., 1984), similar to alterations in fatty acid composition reported in several tissues of streptozotocin diabetic rats (Faas & Carter, 1980; Worcester et al., 1979; Brenner, 1974). Insulin therapy improved glucose tolerance and partially normalized the polyunsaturated fatty acid composition of erythrocytes in these patients (Van Doormaal et al., 1984).

Difficulty exists in studying this form of diabetes because the degree of metabolic control appears to influence insulin action. It appears that insulin therapy (Eaton et al., 1984; Smith, 1980), exercise (Pedersen et al., 1981) and diet (Pedersen et al., 1982b), the three factors used to treat the disorder, exhibit regulatory effects on insulin sensitivity.

Noninsulin Dependent Diabetes Mellitus

Noninsulin dependent diabetes or Type II diabetes is a heterogeneous disorder characterized by defects in both insulin secretion and insulin action (Reaven et al., 1983; Efendic et al., 1984). From the available literature, difficulties exist in characterizing insulin resistance in this form of diabetes; it is a multifactorial disorder manifested by variable degrees of glucose intolerance and different degrees of impaired insulin secretion and action. To attempt to alieviate this problem, the National Diabetes Group of the National Institute of Health (1979) divided noninsulin dependent nonketosis-prone diabetes into two forms, according to whether or not obesity is present. Individuals in these subclasses can then be further characterized by the form of therapy they receive (insulin, oral hypoglycemic agents, diet). A further classification, impaired glucose tolerance, identifies individuals with plasma glucose levels intermediate between those considered normal and those considered diabetic (National Diabetes Data Group, 1979).

Insulin resistance in noninsulin dependent diabetes has been well demonstrated by the use of several *in vivo* techniques (Scarlett et al., 1983; Chen et al., 1987; Del Prato et al., 1983; DeFronzo et al., 1983; Kolterman et al., 1981; Krotkiewski et al., 1985; Mandarino et al., 1984b). *In vivo* studies of glucose metabolism have displayed impaired suppression of hepatic glucose output (DeFronzo et al., 1982; Kolterman et al., 1981) and peripheral glucose utilization (DeFronzo et al., 1982; Kolterman et al., 1981; Scarlett et al., 1983; Chen et al., 1987; Del Prato et al., 1983; Krotkiewski et al., 1985). Reviewing the available literature utilizing *in vivo* techniques to assess insulin action, Truglia et al. (1985) concluded that decreased insulin action predominated in mild chemically-induced diabetes, whereas in moderate to severe glucose intolerance, decreased insulin secretion and action are both present. Both Truglia et al. (1985) and Olefsky & Kolterman (1981) concluded in recent reviews that *in vivo* techniques demonstrate that insulin resistance in target tissues is most extensive in patients who exhibit the greatest degree of carbohydrate intolerance.

Attempts have been made to elucidate the cellular mechanism(s) associated with insulin resistance in noninsulin dependent diabetes. *In vitro* assays of insulin stimulated

glucose metabolism have clearly demonstrated marked alterations in insulin function. Both the maximum response (Scarlett et al., 1983; Ciaraldi et al., 1982; Sinha et al., 1987) and insulin sensitivity (Ciaraldi et al., 1982; Sinha et al., 1987) of adipocytes from diabetic patients were significantly reduced when compared to cells from nondiabetic subjects. The diabetic state markedly impairs the ability of insulin to stimulate adipocyte glucose oxidation (Arner et al., 1987b; Bolinderet al., 1982) and glucose incorporation into lipids (Lonnroth et al., 1983; Krotkiewdki et al., 1985). Glucose metabolism in the absence of insulin was also reported to be reduced in these subjects (Scarlett et al., 1983; Ciaraldi et al., 1982; Lonnroth et al., 1983; Krotkiewski et al., 1985). However, normal basal rates of glucose oxidation were found in another group of diabetic patients (Arner et al., 1987b). The noninsulin dependent diabetic state does not appear to disrupt all cellular function of insulin; the antilipolytic effect of insulin in adipocytes was not reported to differ from that of control cells (Arner et al., 1987b; Lonnroth et al., 1983; Krotkiewski et al., 1985; Scarlett et al., 1983).

Although very few studies measure both *in vitro* and *in vivo* glucose metabolism, those studies that do show that alterations in insulin action in adipocytes from diabetic subjects relate to *in vivo* measure of insulin action (Ciaraldi et al., 1982; Krotkiewski et al., 1985; Scarlett et al., 1983). A positive relationship was reported between maximum insulin stimulated *in vivo* glucose disposal and maximum *in vitro* adipocyte glucose transport rates (Ciaraldi et al., 1982; Scarlett et al., 1983). Changes in glucose incorporation into lipids in adipocytes with physical training was related to glucose disposal, measured by a euglycemic clamp (Krotkiewski et al., 1985).

Based on *in vitro* studies of insulin stimulated action in adipocytes, it has been predicted that both receptor and postreceptor defects coexist in this disease (Scarlett et al., 1983; Ciaraldi et al., 1982; Sinha et al., 1987; Lonnroth et al., 1983). However, there is disagreement concerning the existence of a defect in insulin binding. Studies have reported decreased insulin binding to receptors on adipocytes (Sinha et al., 1987; Kolterman et al., 1981), monocytes (Beck-Nielsen, 1978; Rizza et al., 1981; Mandarino et al., 1984) and partially purified muscle biopsies (Caro et al., 1987) obtained from noninsulin dependent

diabetic patients. In these studies, reduction in the total number of functional receptors is cited as the cause of decreased binding. However, compared to matched control subjects, no alterations in insulin receptor binding to erythrocytes (Comi et al., 1987; Hjollund et al., 1987; Mandarion et al., 1984; Del Prato et al., 1983), monocytes (Hjollund et al., 1987), adipocytes (Arner et al., 1987b; Lonnroth et al., 1983; Bolinder et al., 1982; Hjollund et al., 1987; Krotkiewski et al., 1985) or muscle biopsies (Arner et al., 1987a) were reported. Confusion in the literature as to the existence of a receptor defect in addition to the unclear relationship between alterations in binding and overall insulin action questions the role of the insulin receptor in the well documented defects of insulin action in this form of diabetes. It has been suggested that the cause of insulin resistance might occur primarily at the receptor level when the disease is accompanied by hyperinsulinemia (Olefsky, 1981).

The cellular locus of insulin resistance in noninsulin dependent diabetes may reside beyond the binding portion of the insulin receptor. Recent interest has been focused on the kinase function of the β-subunit of the insulin receptor as either a means for coupling insulin binding to insulin induced effects or as a parallel manifestation of the coupling process that may relate to some of the actions of the hormone. The ability of insulin to induce autophosphorylation of the receptor in partially purified insulin receptors from adipocytes (Friedenberg et al., 1987) and muscle biopsies (Caro et al., 1987) was not altered by the diabetic state. However, noninsulin dependent diabetic subjects compared to nondiabetic subjects demonstrated reductions in insulin stimulated tyrosine-specific protein kinase activity in adipocytes (Sinha et al., 1987), muscle (Arner et al., 1987a; Caro et al., 1987) and erythrocytes (Comi et al., 1987). However, controversy exists as to the role of obesity accompanying the diabetic state in the etiology of the receptor kinase defect (Caro et al., 1987; Arner et al., 1987a; Friedenberg et al., 1987). At present the role of receptor kinase activity in insulin action and intracellular phosphorylation is unknown and the possibility has not been ruled out that the reductions reported in the coupling activity between the receptor and tyrosine kinase activity is not simply the result of some metabolic abnormality associated with the disease. A defect in receptor kinase activity does, however, offer a logical explanation

to clarify the apparent discrepancies reported between insulin binding and action in this form of diabetes.

A number of abnormalities, associated with noninsulin dependent diabetes, have been suggested to contribute to the insulin resistant state. Hyperinsulinemia, both fasting and postprandial, is a characteristic feature of many noninsulin dependent diabetic patients. Inducing hyperinsulinemia in rats decreases insulin binding to adipocytes and causes a decreased sensitivity in insulin stimulated glucose transport which returns to normal when insulin thearapy is stopped (Kobayashi & Olefsky, 1978; York & Singh, 1979). Exposing adipocytes to high concentrations of insulin *in vitro* has been shown to decrease insulin receptor kinase activity (Arsenis & Livingston, 1986). However, there is still limited data to support the concept that hyperinsulinemia is a major factor contributing to insulin resistance in the diabetic state. Fasting insulin levels have not been found to correlate with insulin binding (Lonnroth et al., 1983) and the same degree of adipocyte insulin resistance to glucose oxidation was found in noninsulin dependent diabetic subjects with fasting hyperinsulinemia as in those with normal insulin levels (Arner et al., 1987b).

As many patients with noninsulin dependent diabetes are obese and obesity is a condition associated with insulin resistance (Ciaraldi et al., 1981; Pedersen et al., 1982d), it seems logical that the obese state could contribute to altered insulin action in this form of diabetes. However, insulin stimulated glucose oxidation in obese diabetics did not differ from nonobese diabetics (Arner et al., 1987b). *In vivo* glucose utilization, although markedly reduced when compared to control subjects, did not differ between obese and nonobese noninsulin dependent diabetic subjects (Hollenbeck et al., 1984). Mean adipocyte size was found to correlate with insulin stimulated glucose oxidation in both noninsulin dependent diabetic subjects and obese subjects (Harrison & King-Roach, 1976). However, in this same study, both basal and insulin stimulated oxidation rates were significantly reduced in diabetic subjects compared to obese nondiabetic subjects with a similar cell size (Harrison King-Roach, 1976). As concluded in a recent review (Truglia et al., 1985), obesity may play a role in the development of impaired glucose tolerance but its contribution to insulin resistance

is likely minimal and decreases as hyperglycemia increases.

Therapeutic interventions have been demonstrated to improve insulin action in noninsulin dependent diabetic subjects. Two weeks of intensive insulin therapy, to previously untreated patients, significantly improved both glucose disposal, measured by the euglycemic clamp technique and adipocyte insulin stimulated glucose transport (Scarlett et al., 1983). Although significantly improved after therapy, insulin action in diabetic patients was still less than control subjects (Scarlett et al., 1983). Oral sulphonylurea therapy improved insulin binding to monocytes but remained lower than in nondiabetic subjects (Olefsky & Reaven, 1976). Weight loss by feeding hypoenergetic diets to obese diabetic subjects significantly improved glucose tolerance, determined by intravenous glucose tolerance and insulin stimulation tests (Beck-Nielsen, 1978), and *in vivo* glucose disposal (Zawadzki et al., 1987). In a recent report, weight loss induced by feeding low energy diets for two months to eight obese noninsulin dependent diabetic patients, normalized adipocyte glucose transport, glucose oxidation and lipogenesis (Hjollund et al., 1987). Increasing the fiber and polyunsaturated fat content of the diet has also been demonstrated to improve insulin binding to monocytes (Ward, et al., 1982).

Exercise, another important component in the treatment of diabetes mellitus influences insulin action (Krotkiewski et al., 1985). Placing obese diabetic patients on individualized training programs for three months significantly improved glucose disposal rates, glucose tolerance and normalized insulin stimulated glucose incorporation into adipocyte lipids (Krotkiewski et al., 1985). In this study, unlike other reports on the beneficial effects of therapy, improvements in insulin action occurred without a change in body weight (Krotkiewski et al., 1985).

In conclusion, noninsulin dependent diabetes mellitus is clearly a multifactorial disorder which is manifested by variable degrees of insulin resistance. A postreceptor alteration in insulin action in peripheral tissues appears to be the primary lesion. However, alterations in receptor binding and postbinding receptor-mediated events such as the stimulation of receptor kinase activity may also be present. The degree of insulin resistance is

generally greater in noninsulin dependent diabetes than in subjects with obesity-associated glucose intolerance. Although many noninsulin dependent diabetic subjects are obese, obesity is not the only factor contributing to insulin resistance, as patients of normal weight are, in most cases, also insulin resistant. A partial correction of insulin resistance can occur with therapy aimed at improving diabetic control.

Streptozotocin Diabetic Rat

The streptozotocin diabetic rat is one of the best studied of the currently available animal models for insulin action in the diabetic state. Streptozotocin (2-deoxymethyl-nitrosourea-glucopyranose), a metabolite of the soil organism *Streptomyces alcromogenes*, was first reported to be diabetogenic in rats and dogs in 1963 (Rabieten et al., 1963). Once injected, the drug induces selective destruction of the beta cells of pancreatic islets by damaging the permeability of the plasma membrane such that the ability to secrete insulin in response to glucose is severely attenuated in animals within 24 hours after drug administration (Bell & Hye, 1983).

Adipocytes from streptozotocin diabetic rats respond poorly to insulin *in vitro*. The capacity in both the basal and insulin stimulated states to oxidize glucose (Schoenle et al., 1977; Kobayashi & Olefsky, 1979; Sandra & Fyler, 1982) and incorporate glucose into lipids (Schoenle et al., 1977; Kobayashi & Olefsky, 1979) is significantly impaired. In this diabetic model, there is also a marked decrease in insulin's ability to stimulate adipocyte glucose transport (Schoenle et al., 1977; Kobayashi & Olefsky, 1979; Sandra & Fyler, 1982), protein synthesis (Tischler et al., 1986) and to inhibit epinephrine induced lipolysis (Zapf et al., 1975). Basal rates of glucose transport have been reported to be both increased (Schoenle et al., 1977) and decreased (Karnieli et al., 1981b; Sandra & Fyler, 1982) in this syndrome. Impairment of insulin action in adipocytes from diabetic rats is directly related to the severity of the diabetic state (Kobayashi & Olefsky, 1979). Even when corrected for cell size by expressing data per unit surface area, glucose metabolism remained clearly reduced in diabetic adipocytes (Kobayashi & Olefsky, 1979).

Insulin stimulated glucose transport in rat adipocytes occurs primarily through a translocation of glucose transporters from a large intracellular pool to the plasma membrane (Kono et al., 1981). In the streptozotocin diabetic rat, resistance to the stimulatory effect of insulin on glucose transport has been demonstrated to be caused by a decreased translocation of glucose transporters to the plasma membrane as a consequence of depletion of transporters in the intracellular pool (Kahn & Cushman, 1987; Karnieli et al., 1981b).

A poor metabolic response to insulin might be explained by alterations in the binding of insulin to its cellular receptor. However, the literature indicates that this is not the case; insulin binding is significantly increased in adipocytes from streptozotocin diabetic rats (Schoenle et al., 1977; Kobayashi & Olefsky, 1979; Sandra & Fyler, 1982). The magnitude of the enhanced receptor binding increases with the severity of the diabetic state (Kobayashi & Olefsky, 1979). This reported increase in insulin receptor binding is consistent with the concept of Gavin et al. (1974) that circulating insulin levels inversely regulate insulin receptors. Even if this is the case, the enhanced binding is not great enough to compensate for the metabolic defects in insulin action caused by insulin insufficiency in this model.

The mechanism for insulin resistance in this form of diabetes is not completely understood. Streptozotocin-induced diabetes has been associated with a reduction in insulin stimulated receptor kinase activity (Gherzi et al., 1986; Kadowaki et al., 1984) that improve with insulin therapy (Kadowaki et al., 1984). However, normal kinase activity has been reported (Amatruda & Roncone, 1985). Descrepancies in the relationship between kinase activity and the diabetic state may relate to differences in cell prepartions used. A reduced activity of five of the enzymes involved in adipose tissue triglyceride synthesis (fatty acyl-CoA synthetase, mitochondrial and microsomal glycerolphosphate acyltransferase, monoacylglycerol-phosphate acyltransferase and phosphatidate phosphohydrolase) were reported in the streptozotocin diabetic rat (Saggerson & Carpenter, 1987). Insulin administration, both *in vitro* and *in vivo*, was shown to increase the activities of these enzymes (Saggerson & Carpenter, 1987).

The diabetic condition produces considerable alterations in the fatty acid profile of tissues. Early studies, utilizing diabetic animals, indicated alterations in the monounsaturated and polyunsaturated fatty acyl composition of various tissue lipids (Benjamin & Gellhorn, 1964; Brenner et al., 1968; Friedman et al., 1966). Reduced levels of monounsaturated fatty acids and arachidonic acid ($C_{20:4(6)}$) in liver and adipose tissue have been related to diminished rates of hepatic $\Delta 9$ and $\Delta 6$ desaturase in diabetic animals (Faas & Carter, 1980; Worchester et al., 1979; Brenner, 1974). The decrease in desaturase activities associated with the diabetic state suggests that these enzymes are insulin dependent. Insulin therapy corrects the defects but only partially normalizes the altered fatty acid composition, as arachidonic acid levels remain reduced (Faas & Carter, 1980). High fat diets, especially those high in polyunsaturated fats, have been shown to inhibit $\Delta 9$ and $\Delta 6$ desaturation (Garg et al., 1988; Weekes et al., 1986). However, due to the use of experimental diets differing in level and type of fatty acids and the use in some studies of essential fatty acid deficient diets, the possible role of diet in modifying diabetes-associated changes has not been elucidated. The effect of the diabetic state on membrane lipid composition of insulin sensitive tissues and the physiological consequences of reported alterations remain to be determined. There is one report suggesting that membranes from diabetic animals may respond differently to lipid alterations. Treating adipocytes from control animals with phospholipid vesicles composed of dioleoylphosphatidylcholine and phosphatidylserine markedly inhibited insulin stimulated glucose transport and oxidation in control adipocytes (Sandra & Fyler, 1982). Similar treatment to adipocytes obtained from streptozotocin diabetic rats did not further reduce the already significantly lower levels of glucose metabolism (Sandra & Fyler, 1982). In this study, the effect of phospholipid modifications on normal adipocytes produced similar effects to those observed in cells from untreated diabetic animals, because glucose uptake and oxidation were inhibited in such a way that even supraphysiological concentrations of insulin did not restore normal responsiveness (Sandra & Fyler, 1982).

Although the role of diet therapy has not been extensively studied in this model, recent evidence suggests high polyunsaturated fat diets may be beneficial. In

streptozotocin-diabetic rats, there is an enhanced intestinal uptake of many nutrients, including sugars, amino acids and lipids (Thomson, 1983). Small changes in the lipid composition of the diet have been shown to influence active and passive intestinal transport in the rat (Thomson et al., 1986). Recently it was demonstrated that feeding a polyunsaturated diet diminished the enhanced jejunal and ileal uptake of glucose associated with the diabetic condition (Thomson et al., 1987). In addition, feeding a high fat high P/S diet as compared to a low P/S diet, significantly improved the clinical condition of diabetic animals as determined by lower hemoglobin A1C levels (Rajotte et al., 1988).

The streptozotocin-diabetic rat has been used as a model of both human noninsulin dependent and insulin dependent diabetes. Whether insulin deficiency, the primary cause of insulin resistance in this model, is a major factor leading to insulin resistance in human diabetes is not clear. At present there are no animal models, possibly due to the heterogeneous nature of the disease, completely representative of noninsulin dependent diabetes. However, *in vitro* resistance to action of insulin on the multiple steps of glucose metabolism in this animal model closely resembles that reported in individuals with noninsulin dependent diabetes.

G. CONCLUSIONS

Insulin binding to its membrane is not a constant biological process as it now appears that both the number and affinity of insulin receptors are subject to dynamic regulation. Less, however, is known about the complex processes through which insulin receptor binding leads to the biological responses. Resistance to the action of insulin plays a central role in many disease states, including diabetes and obesity. The composition of the diet, in particular the fat/carbohydrate content, has been demonstrated to influence insulin action. Difficulties exist in interpreting these dietary changes, because researchers have altered several dietary components simultaneously or have fed animals diets not representative of those consumed by the human population. Based on *in vitro* manipulation, the membrane lipid environment appears to play an important role in determining insulin binding, glucose transport and the generation of intracellular mediators of insulin action. As diet therapy is an important

component of the treatment of insulin resistant states of diabetes and obesity, further research is required to identify the role of diet, in particular diet fat composition, on insulin binding and action.

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II. RESEARCH PLAN

A. RATIONALE

Diet has an overall effect on insulin action. However, as most comparisons studied in this regard are not representative of the human diet with respect to the content and compostion of diet fat, the role of dietary fats on cellular insulin binding and action remains unclear. The plasma membrane is an important factor in insulin action because it provides the interface for the initial contact of the hormone with the cell through binding of the ligand to a membrane receptor and, through a variety of mechanisms, has been postulated to be important in generating the cellular actions of insulin. It has been clearly demonstrated *in vitro* that the activities of the insulin receptor and glucose transporter are sensitive to the physical and chemical properties of membrane lipids. Although, for a variety of membranes, diet fat-induced alterations in membrane lipid composition have been demonstrated to alter the function of integral membrane proteins, the effect of diet fat on the adipocyte plasma membrane lipid composition and the action of insulin is not known. Several disease states, such as diabetes and obesity, where diet is a major component of therapy, are characterized by cellular resistance to insulin action. Thus, an understanding of the role of diet-induced alterations on membrane composition and cellular insulin action may be important to the fundamental understanding of the role of diet and membrane in hormonal action in the normal state and is relevant to developing effective dietary strategies with a mechanistic basis for management of disease states.

B. OBJECTIVES

The objectives of this thesis research are to demonstrate that:

1. a relationship exists between the composition and content of dietary fat and the lipid composition of the adipocyte;
2. the composition and content of dietary fat influences the cellular insulin action;
3. a relationship exists between diet fat induced alterations in membrane lipid composition

and membrane mediated events of insulin action; and

4. the diabetic state will modify the relationship between diet fat, membrane composition and insulin function.

C. HYPOTHESES

It is specifically hypothesized that:

In both the normal and diabetic states, differences in content and composition of fat in the diet will be reflected in:

1. the stored triglyceride content of the adipocyte;
2. the phospholipid content of the adipocyte plasma membrane; and
3. the fatty acyl compositon of the major phospholipids of the adipocyte plasma membrane.

In both the normal and diabetic states, the composition of dietary fat will alter:

4. insulin binding;
5. glucose transport; and
6. intracellular glucose metabolism.

It is further hypothesized that:

7. diet-induced alterations in the fatty acyl composition of the adipocyte plasma membrane will relate to the integral membrane function of insulin binding.

D. CHAPTER FORMAT

The hypotheses posed were tested in a sequence of experiments. These experiments are organized as thesis chapters and have been submitted for scientific publication as individual papers.

Chapter III examines the effect of dietary polyunsaturated to saturated fatty acid (P/S) ratio on the fatty acyl composition of the major membrane phospholipids of the adipocyte plasma membrane (hypothesis 2). Two high fat diets (20% w/w) providing a P/S ratio of 0.25 or 1.0, representative of those consumed by segments of the North American population, were fed for 4-5 weeks to 6 week old control and streptozotocin-induced diabetic

rats. The effect of diet P/S on insulin binding, an integral membrane function, was assessed (hypothesis 4).

In Chapter IV, the effect of the same dietary design and experimental procedures reported in Chapter III on insulin stimulated intracellular glucose metabolism (glucose oxidation and glucose incorporation into lipids) was assessed (hypothesis 6).

In Chapter V, the effect of dietary P/S on insulin stimulated membrane mediated glucose transport (hypothesis 5) and intracellular glucose metabolism (hypothesis 6) in the normal and diabetic states were examined further by altering the P/S ratios (0.2 and 2.0) to reflect dietary extremes consumed by the human population. Diets were fed to weanling rats for 6 weeks. The effect of glucose concentration on insulin stimulated glucose transport at several insulin concentrations was also assessed for each diet/treatment group (hypothesis 5).

In Chapter VI, the effect of the diet P/S on the fatty acyl composition of the major phospholipids of the adipocyte plasma membrane for the animals described in Chapter V was determined (hypothesis 3).

In Chapter VII, the effect of both the content (10% w/w and 20% w/w) and composition (P/S=0.2 and P/S=2.0) of diet fat on the content (hypothesis 2) and composition (hypothesis 3) of the major adipocyte membrane phospholipid fractions in control and diabetic animals was determined under the same experimental conditions as reported in Chapter V.

In Chapter VIII, the effect of fat content and composition on insulin stimulated glucose transport (hypothesis 5) and intracellular glucose metabolism (hypothesis 6) in control and diabetic states was determined. A high fat (20% w/w) low P/S (0.2) and two low fat (10% w/w) diets providing a P/S ratio of 0.2 and 2.0 were fed. The same experimental conditions as reported in Chapter V were used.

In Chapter IX, the relationship between dietary P/S, membrane composition and insulin binding was determined (hypothesis 7) by feeding control animals one of ten high fat diets (20% w/w) with P/S ratios ranging from 0.14 to 1.8.

In Chapter X, the effect of the content and composition of the diets described in Chapter VII on the fatty acyl compositon of stored lipids in adipocytes was determined in control and diabetic animals (hypothesis 1).

Discussion of the potential relationships between diet-induced change in adipocyte membrane composition and insulin receptor mediated function(s) is summarized and drawn together in Chapter XI.

III. DIETARY FAT AND THE DIABETIC STATE ALTERED INSULIN BINDING AND FATTY ACID COMPOSITION OF ADIPOCYTE PLASMA MEMBRANE [1]

A. INTRODUCTION

There is convincing evidence in animal models for several tissue types that physiological changes in the type of diet fat consumed changes cell membrane composition (Clandinin et al., 1985) by mechanisms that can involve altering activity of enzymes of fatty acid desaturation (Garg et al., 1988) and the *de novo* biosynthesis of membrane phosphatidylcholine (Hargreaves & Clandinin, 1987a; Hargreaves & Clandinin, 1987b). These diet-induced alterations in membrane phospholipid composition influence the function of membrane proteins by changing the lipid environment in which these proteins act (Clandinin et al., 1985). It has also been established in man and animals that normal variation in dietary fatty acid intake can alter the fatty acid composition of adipose tissue triglycerides and phospholipids (Field & Clandinin, 1984; Carroll, 1965; Ostwald et al., 1962). However, little is known about the role of the adipocyte plasma membrane phospholipid and the influence of physiological shifts in fat intake or disease state on the composition and function of this membrane. Recently, a relationship between habitual fatty acid intake and the fatty acid composition of triglycerides and phospholipids in human adipose tissue was established (Field et al., 1985), suggesting that long term intake of fat may determine the membrane lipid composition in this major organ.

Noninsulin dependent diabetes is characterized by a cellular resistance to insulin action (Olefsky & Reaven, 1977). Although the mechanisms for insulin resistance are not completely understood, it has been suggested to be the consequence of reduced insulin binding is due to a reduction in functional insulin receptors (Pedersen, 1984). Although alterations in insulin binding to adipocyte membrane receptors have also been shown to be altered in this form of diabetes (Kolterman et al., 1981; Pedersen, 1984), controversy exists in the literature as to the existence of a receptor defect in noninsulin dependent diabetes (Arner et al., 1987; Lonnroth

[1]A version of this chapter has been accepted for publication. Field CJ, Ryan EA, Thomson ABR & Clandinin MT. (1988) Biochem J

et al., 1983).

The insulin receptor is embedded in the lipid bilayer of the plasma membrane (Schlessinger et al., 1978). *In vitro*, the insulin receptor (Ginsberg et al., 1981; Gould et al., 1982) and insulin function (Sandra et al., 1984) appear sensitive to the surrounding lipid environment. The nature of fat fed has been reported to influence insulin binding to receptors in erythrocytes (Gould et al., 1982) and Ehrlich ascites cells (Ginsberg et al., 1982), but whole body physiological tests of this relationship have not been reported, with one exception. When a high polyunsaturated diet was fed to diabetic rats, diet-induced alteration in the form and function of the intestine have been reported to improve the clinical state in this diabetic model (Rajotte et al., 1988).

The diabetic state may also influence the fatty acid composition of cell membranes. Reduced levels of monounsaturated fatty acids and polyunsaturated fatty acids in liver and adipose tissue have been related to diminished microsomal rate of $\Delta 6$ and $\Delta 9$ fatty acid desaturation in experimental models of diabetes mellitus (Faas & Carter, 1980; Worcester et al., 1979; Benjamin & Gellhorn, 1964; Brenner et al., 1968; Friedman et al., 1966). Insulin therapy appears to correct these defects in desaturase activity and to partially normalize the altered fatty acid composition of microsomes (Faas & Carter, 1980). The physiological importance of these alterations is not clear.

Based on the premise that the composition of the adipocyte plasma membrane is an integral determinant of some aspects of insulin stimulated functions in the adipocyte, the following study was designed to examine the effect of alterations in dietary fatty acid composition on the fatty acid composition of membrane phospholipids and on insulin binding to its receptor on the adipocyte plasma membrane receptor. Dietary transitions representing the physiological range of dietary fatty acid composition consumed by humans were used. Control and diabetic rats were compared to assess whether increasing consumption of polyunsaturated fatty acids would normalize reduced levels of desaturase products anticipated in the diabetic state and would alter insulin binding, an integral membrane function that could regulate insulin responsiveness of this tissue.

B. MATERIALS AND METHODS

Animal and Diets

Forty male Sprague-Dawley rats, 4 weeks of age (140±10g) were randomly assigned to two groups and fed a semi-purified high fat diet containing 27% (w/w) high protein casein, 38% (w/w) carbohydrate as cornstarch and 20% (w/w) fat. The diet was supplemented with the following per kg: 2.75 g choline, 6.25 g inositol, 2.5 g L-methionine, 80 g cellulose, 10 g vitamin mix (A.O.A.C.), and 50 g Bernhart-Tomerelli mineral mix. The composition of this basal diet, vitamin and mineral mixes has been reported in detail earlier (Hargreaves & Clandinin, 1987b). The two diets fed differed only in the proportion of safflower oil and hydrogenated beef tallow fed, to provide a polyunsaturated to saturated fatty acid ratio (P/S) of 0.25 (low P/S) or 1.0 (high P/S) as illustrated (Table III-1). Linseed oil was added to both diets to obtain a total *w*-3 fatty acid content of 1% (w/w) of the diet fat. Both diets were cholesterol-free by analysis. Animals were housed individually in a temperature controlled room at 21°C±1°C and maintained on a 12 hour light/dark cycle.

After feeding the diet for 14 days, diabetes was induced in half of the animals in each diet group by intravenous injection of streptozotocin (65 mg/kg body weight; Sigma Chemical Co., St. Louis, MO, U.S.A) in a citrate buffer pH 4.5 into a tail vein. Only animals with non-fasting blood glucose levels greater than 300 mg/100 ml after 14 days were considered diabetic. Animals were continued on their respective diet treatment for an additional 14 to 24 days.

Each day, at 0900 h (two hours after the end of the dark cycle) for a period of 10 days an animal from each treatment group was sacrificed by decapitation and serum collected for glucose and insulin determinations. Serum glucose and insulin were determined using a glucose analyzer (Glucose Analyzer 2, Beckman Instruments, Inc., Fullerton, CA) and by radioimmunoassay (Insulin RIA 100 Kit, Pharmacia Co., Uppsala, Sweden) against a rat insulin standard. Epididymal fat pads were collected and adipocytes isolated by incubation with a crude collagenase (2.25 mg/g tissue; Cooper Biomedical, Toronto) by a modification of

the method of Rodbell (1964). The modification included the use of a glucose-free Krebs-Ringer solution buffered with Hepes (Whitesell & Abumrad, 1985) containing 2% (w/v) Fraction V bovine albumin (Sigma Chemicals, St. Louis, MO, U.S.A.).

Plasma Membrane Isolation

Plasma membranes were prepared from adipocytes according to the method of Lewis et al. (1979) modified to enable isolation of plasma membranes from small samples of material in 1 ml using a Beckman TL-100 ultracentrifuge (Beckman Instruments Ltd., Palo Alto, California). The modification included loading the second pellet, resuspended in 200 μl buffer (0.25M sucrose, 1mM EDTA, 10mM Tris-HCl) at pH 7.4, on an 800 μl linear gradient containing 32-52% (w/v) sucrose, 1mM EDTA and 10mM Tris-HCl, pH 7.4, followed by centrifugation at 45,000 rpm (205,780 x g at r_{ave}) in a Beckman TLS-55 rotor for 30 min at 4°C. The white band near the top of the gradient containing the plasma membrane fraction was collected and diluted to a volume of 1 ml in 10mM Tris-HCl, 1mM EDTA buffer (pH 7.4) and centrifuged at 29,000 rpm (30,000 x g at r_{ave}) for 30 min at 4°C in a Beckman TL-100 rotor. The membrane pellet was collected, resuspended in 400 μl 10mM Tris-HCl buffer and stored at -70°C for analysis of lipid composition. Purity of this plasma membrane fraction was assessed by assaying 5'nucleotidase (Goldfine et al., 1977) and succinate dehydrogenase (Kun & Abood, 1949) in membrane fractions obtained from control animals fed the high fat diets (Appendix I, Table A-1). The specific activity of 5'nucleotidase was highest in the plasma membrane fraction (6.46$\pm$0.04 μmoles phosphorus hydrolyzed/mg protein/hour) representing greater than eight-fold enrichment from cell homogenate. Succinate dehydrogenase activity was low in the plasma membrane (1.47$\pm$0.12 μmoles para-iodonitrozolium violet reduced/mg protein/hour) representing less than 11% of the activity found in the mitochondrial fraction. The purity of the plasma membrane fraction was comparable to previous reports (Lewis et al., 1979) and not affected by diet treatment or the diabetic state (Kahn & Cushman, 1987).

Membrane Lipid Analysis

Lipids were extracted from plasma membranes by a modified Folch procedure (Folch et al., 1957). This extraction procedure was modified as follows: to 400 µl Tris-HCl buffer pH 7.4 containing the membrane pellet, 0.8 ml methanol, 2.0 ml chloroform:methanol (1:1 v/v), 2.7 ml chloroform and 2.5 ml chloroform:methanol (2:1 v/v) were added in succession. The layers were split by addition of 1.6 ml of 0.1 mM KCl. The lower phase containing the phospholipids was collected. Individual phospholipids were separated on Whatman HP-K thin layer chromatography plates (10 x 10 cm) using the following solvent system: chloroform:methanol:2-propanol:0.25% (w/v) KCl:triethylamine (30:9:25:6:18 by vol.) (Touchstone et al., 1980). Separated phospholipids were sprayed with 0.03% w/v 2'7'dichlorofluorescein in 0.01 M NaOH and detected by comparison under u.v. light with appropriate standards.

Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin fatty acid methyl esters were prepared using 14% w/v BF_3/methanol reagent (Morrison & Smith, 1964) and separated by automated gas-liquid chromatography (Vista 6010 G.L.C. and Vista 402 data system; Varian Instruments, Georgetown, Ontario, Canada). Chromatography was performed using a fused silica BP20 capillary column (25m x 0.25mm I.D.; Varian, Georgetown, Ontario, Canada). Helium was used as the carrier gas at a flow rate of 1.8 ml/min using a splitless injection mode. The initial oven temperature was 150°C, increased to 190°C at 20°C/min and held for 23 mins, then increased to 220°C at 2°C/min for a total analysis time of 40 mins. The analytical conditions used separate all saturated, mono-, di-, and polyunsaturated fatty acids from C_{14} to C_{24} carbons in chain length.

Insulin Binding

Freshly isolated adipocytes were incubated with ^{125}I-porcine insulin (New England Nuclear, Boston, MA, U.S.A.) having a specific activity of 89.3 µCi/µg in the buffer described for cell isolation at 24°C pH 7.4 as described by Olefsky & Reaven (1975) in a total

incubation volume of 600 μl. Optimal steady-state binding conditions were achieved for both control and diabetic adipocytes at 24°C after 45 min of incubation and were maintained for at least 1 additional hour (Appendix Figure A-1). Insulin binding was terminated as described by Gammeltoft & Gliemann (1973) by rapidly centrifuging 250 μl aliquots from cell suspensions in polyethylene microcentrifuge tubes to which 100 μl silicon oil had been added in a MSE microcentrifuge (John's Scientific, Toronto, Ont., Canada) on high speed (570 g x 1 min). The cells were collected, added to 5 ml Aquasol (New England Nuclear) and radioactively measured in a Beckman LS 5801 liquid scintillation counter at a counting efficiency of 90% as determined by direct counting in a γ counter. Binding was corrected for non-specific binding by incubating cells with ^{125}I-insulin and 20 μg/ml unlabelled insulin. The nonspecific binding was 5-10% of total binding and did not vary with diet treatment or disease state. Cell numbers were determined by 24 hour fixation at 36°C in 2% osmium tetroxide in 0.05M collidine (made in 0.15M saline) as described by Hirsch & Gallian (1968) using a Coulter ZM counter and 400 μm aperture (Coulter-Electronics, Vancouver). Binding was calculated on a per cell basis and expressed as total amount of insulin bound per 2.0 x 10^5 cells.

Statistical Analysis

The effect of diet treatment and disease state on membrane phospholipid fatty acid composition and insulin binding was compared by leasted-squared analysis of variance procedures (Harvey, 1975). A Duncan's multiple range test was used to discriminate significant differences between individual treatment groups (Steel & Torrie, 1980).

C. RESULTS

Animals Weight, Serum Glucose and Serum Insulin

The diabetic animals weighed less, and exhibited higher serum glucose levels and lower serum insulin levels, compared with control animals (Table III-2). Dietary lipid modification did not significantly alter these three parameters in either control or diabetic animals.

Although food consumption was not measured, it has been well documented by others that diabetic animals display hyperphagia (Kobayashi & Olefsky, 1979).

Effect of Diet on Adipocyte Plasma Membrane Fatty Acyl Composition

In the present study, diet significantly ($p<0.05$) altered the fatty acid composition of the major adipocyte plasma membrane phospholipids in both control and diabetic animals (Table III-3). Feeding a high polyunsaturated fat diet (P/S=1.0) as compared with a low polyunsaturated fat diet (P/S=0.25) increased the total polyunsaturated fatty acid content (Figure III-1A) and P/S ratio (Figure III-1B) in all five membrane phospholipids analyzed from control animals. For example, consumption of the high P/S diet by control animals increased the content of $C_{20:4(6)}$ in phosphatidlyethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin (Figure III-1C), the content of $C_{18:2(6)}$ in phosphatidylcholine and phosphatidylethanolamine and the total number of double bonds expressed as unsaturation index in fatty acids found in phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine (Table III-3).

In diabetic animals, the tendency to higher phospholipid polyunsaturated fatty acid content in animals fed the high P/S diet was also evident (Figure III-1). Feeding the high P/S diet compared with the low P/S diet to diabetic animals increased the polyunsaturated fatty acid content in phosphatidylserine and phosphatidylinositol (Figure III-1A), P/S ratio in phosphatidylethanolamine and phosphatidylinositol (Figure III-1B) and the unsaturation index of phosphatidylinositol and phosphatidylethanolamine (Table III-3). A higher content of $C_{18:2(6)}$ in phosphatidylserine (Table III-3) and $C_{20:4(6)}$ in sphingomyelin (Figure III-1C) was observed in plasma membranes obtained from diabetic animals fed the high P/S diet compared with the low P/S diet.

Effect of Diabetic State on Adipocyte Plasma Membrane Fatty Acid Composition

The diabetic state significantly influenced both the fatty acid composition of and the degree of diet-induced alterations occurring in the fatty acyl content of the adipocyte plasma

membrane (Table III-3). In the present study, the influence of diet on the polyunsaturated fatty acid content of membrane phospholipids was attenuated by the diabetic state, particularly in phospholipid classes obtained from animals fed the high P/S diet. Membranes from diabetic animals fed the high P/S diet exhibited significantly lower total polyunsaturated fatty acid content (Figure III-1A), P/S ratio (Figure III-1B) and change in unsaturation index for phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Table III-3) compared with control animals fed the same diet. The total polyunsaturated fatty acid content and the P/S ratio in phosphatidylcholine, phosphatidylserine and phosphatidylinositol in diabetic animals fed the high P/S diet was decreased to a level that was not significantly different from control animals fed the low P/S diet.

With the exception of $C_{20:4(6)}$ in phosphatidylcholine (Figure III-1C), the total polyunsaturated, P/S ratio and $C_{20:4(6)}$ content of membrane phospholipids of diabetic animals fed the low P/S diet did not differ significantly from diet-matched control animals (Figure III-1). Significantly lower levels of arachidonic acid were found in phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine obtained from diabetic animals fed the high P/S diet than observed for diet-matched control animals (Figure III-1C). In diabetic animals compared with control animals fed low P/S diet, a significantly lower level of $C_{20:4(6)}$ was found in phosphatidylcholine (Figure 1C).

Effect of Diet on Insulin Binding in Normal and Diabetic Animals

At all insulin concentrations used to test insulin binding, cells from control animals fed the high P/S diet bound significantly more insulin than cells from control animals fed the low P/S diet (Figure III-2). Scatchard analysis (Figure III-2) of binding in control animals suggests that increased insulin binding and is associated with an increased number of available insulin binding sites. An effect of diet on insulin binding was not observed when comparing adipocytes obtained from diabetic animals. At the lower insulin concentrations examined (0.1-10ng/ml), the diabetic adipocytes bound significantly more insulin than adipocytes obtained from control animals fed the low P/S diet (Figure III-2). However, at higher insulin

concentrations (50-100ng/ml) tested, cells from diabetic animals bound significantly less insulin than control cells from animals fed the high P/S diet, but the amount of insulin bound at these levels did not differ from cells from control animals fed the low P/S diet.

D. DISCUSSION

The present study demonstrated that feeding diets providing a fat content and fatty acid composition analogous to that consumed by segments of the North American population (Field et al., 1985) significantly altered the fatty acyl composition of the major phospholipids of the adipocyte plasma membrane in both control and diabetic animals. Individual phospholipid classes responded to dietary fatty acid manipulation to different degrees (Table III-3). Although the molecular control mechanisms through which dietary fat composition influences the fatty acyl composition are unknown, differences in: rates of *de novo* synthesis of phospholipids (Hargreaves & Clandinin, 1987a); redistribution of fatty acyl chains via phospholipase (Van den Bosch, 1980) or acyl transferases (Hargreaves & Clandinin, 1987b); and direct desaturation of membrane phospholipid-linked fatty acids (Garg et al., 1988) have been demonstrated to be altered by dietary fat composition. The availability of fatty acids at specific sites of synthesis may be influenced by dietary fat as adipose tissue represents a large pool of available fatty acids, of which the composition is to a large extent reflective of the dietary fatty acid composition (Field & Clandinin, 1984; Carroll, 1965; Ostwald et al., 1962).

The present study demonstrated that induction of the diabetic state can rapidly alter the composition of adipocyte membranes in a manner that can to some degree be attenuated by dietary treatment. Drug-induced diabetes has been shown to decrease the synthesis of polyunsaturated fatty acids, a process which correlated to decreased activity of insulin dependent microsomal $\Delta 9$ and $\Delta 6$ desaturation in liver (Faas & Carter, 1980; Worcester et al., 1979; Benjamin & Gellhorn, 1964; Brenner et al., 1968; Friedman et al., 1966). Insulin therapy has been shown to increase desaturase activity ($\Delta 9$ and $\Delta 6$) and correct alterations in the fatty acyl composition, with the notable exception of lower levels of $C_{20:4(6)}$ (Faas & Carter, 1980). The present study supports the finding of lower total polyunsaturated fatty

acid and $C_{20:4(6)}$ content in most of the major adipocyte phospholipids from diabetic animals fed the high P/S diet compared to their diet-matched controls. In the case of the low P/S diet, the total polyunsaturated fatty acid and $C_{20:4(6)}$ composition, with the exception of the lower arachidonic acid content in phosphatidylcholine, were not significantly reduced by the diabetic state. However, a clear diet effect was observed between the polyunsaturated fatty acid composition of the diabetic animals fed the high and low P/S diets. In the present study, the diabetic state did not produce the dramatic decreases in monounsaturated fatty acids reported by others (Faas & Carter, 1980; Worcester et al., 1979), with the exception of reduced levels of monounsaturated fatty acids in phosphatidylinositol from diabetic animals fed the low P/S diet. It is conceivable that feeding high fat diets in the current experiment, which provided an adequate content of essential fatty acids, may have prevented this diabetes-induced change observed in monounsaturated fatty acids. The greater effect of diabetes on membranes of diabetic animals fed the high P/S diet might be partially explained by the observation that high polyunsaturated fat intakes inhibit $\Delta 9$ and $\Delta 6$ desaturase activity (Garg et al., 1988; Weekes et al., 1986).

The finding that a high polyunsaturated fat diet improved insulin binding to control adipocytes supports earlier work *in vitro* utilizing cell cultures (Ginsberg et al., 1981), reconstituted systems (Gould et al., 1982; Pilch et al., 1980) and *in vivo* growing tumour cells in mice fed different dietary fats (Ginsberg et al., 1982) to alter membrane lipids and insulin binding. Scatchard analysis of binding in control animals is in agreement with the finding of Ginsberg et al. (1982), suggesting a more polyunsaturated environment is associated with an increased number of available binding sites. The molecular mechanisms by which a more polyunsaturated membrane environment improves insulin binding are not clear, but to date several hypotheses have been proposed. The assessibility of the insulin receptor for its ligand may require conformational changes in the receptor that require a more polyunsaturated environment (Gould et al., 1982), or alterations in the quaternary structure or state of the receptor that are determined by the level of membrane saturation (Ginsberg et al., 1982). Membrane alterations have also been suggested to alter insulin action post binding by altering

the mobility of the receptor within the lipid bilayer (Melchior & Czech, 1979), changing the activity of the enzymes involved in phosphatidylinositol turnover (Takenawa & Nagai, 1982) and decreasing the generation of a mediator produced by insulin stimulation from the plasma membrane that mimics the effect of the hormone on a variety of intracellular enzymes (Jarett & Kiechle, 1984).

Unlike cells from control animals, dietary fat treatment did not alter the amount of insulin bound by cells from diabetic animals. Despite clear resistance to insulin action, compared to control cells, insulin binding to adipocytes has been reported to be significantly increased in the streptozotocin diabetic animal fed a high carbohydrate low fat diet at both high and low insulin levels (Schoenle et al., 1977; Kobayashi & Olefsky, 1979; Sandra & Fyler, 1982). This increase in binding is consistent with the concept that circulating insulin levels inversely regulate insulin receptors (Gavin et al., 1974). The results of the present study suggest that feeding a high fat diet may change the comparison between insulin binding in diabetic animals and control animals. At the lower insulin levels, cells from diabetic rats bound more insulin than cells from control animals fed the low P/S diet. However, at higher insulin concentrations where preliminary results indicate a clear defect in intracellular insulin action (Field et al., 1987), cells from diabetic rats bound significantly less insulin than cells from control animals fed the high P/S diet.

The present study demonstrates that normal variations in dietary fat intake: 1) alter the composition of structural lipids found in the epididymal adipocyte; and 2) in the short-term, the diabetic state alters the membrane response to a high polyunsaturated fatty acid diet. These diet-induced alterations in membrane composition appear to influence insulin binding to adipocyte membranes. It is therefore logical to hypothesize that localized changes in the adipocyte plasma membrane microenvironment may play some role in modulating insulin action within cells and may be of particular importance in dietary management of diabetes.

Table III.1 Fatty acid composition of diets.

Fatty Acid	P/S = 1.0	P/S = 0.25
$C_{14:0}$	1.3	2.7
$C_{14:1}$	0.1	0.1
$C_{15:0}$	0.2	0.4
$C_{16:0}$	16.1	22.0
$C_{16:1}$	0.2	0.3
$C_{17:0}$	0.9	1.6
$C_{18:0}$	27.5	47.1
$C_{18:1}$	8.0	4.2
$C_{18:2(6)}$	43.8	18.2
$C_{18:3(6)}$	0.2	0.3
$C_{18:3(3)}$	0.9	0.9
$C_{20:0}$	0.4	0.7
$C_{20:1}$	0.1	0.1
$C_{20:3(6)}$	0.1	0.1
$C_{22:0}$	0.2	0.2
$C_{22:6(3)}$	0.1	0.1
Σpolyunsats	45.0	19.5
Σ(*w*-6)	44.0	18.5
Σ(*w*-3)	1.1	1.0
Σsats	45.7	74.7
Σmonounsats	8.3	4.6
P/S ratio	0.99	0.26

Fatty acid composition of diets fed is expressed as % (w/w) of total fatty acids and was determined by gas liquid chromotography. Abbreviations used: Σpolyunsats, sum of polyunsaturated fatty acis; Σ(*w*-6), sum of (*w*-6)-unsaturated fatty acids; Σ(*w*-3), sum of (*w*-3)-unsaturated fatty acids; Σsats, sum of saturated fatty acids; Σmonounsats, sum of monounsaturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio.

Table III.2 Animal final body weight, serum glucose and insulin levels.

Group	Diet	Final Body Weight (g)	Serum Glucose (mg/100 ml)	Serum Insulin (mU/l)
Control	P/S = 1.0	387 ± 10^{a}	141 ± 3^{a}	61 ± 9^{a}
Control	P/S = 0.25	364 ± 9^{a}	133 ± 3^{a}	55 ± 7^{a}
Diabetic	P/S = 1.0	301 ± 12^{b}	514 ± 33^{b}	28 ± 4^{b}
Diabetic	P/S = 0.25	288 ± 6^{b}	501 ± 36^{b}	23 ± 3^{b}

Values are group mean ± S.E. ($n=10$). Treatments without a common superscript are significantly different ($p<0.05$). 1 μg insulin = 25 mU.

Table III-3. Effect of diet fat and the diabetic state on the major fatty acid content of adipocyte plasma membrane phospholipids.

Phospholipid	Diet	P/S Ratio	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2(6)}$	Σsats	Σmonounsats	(ω-6)	(ω-3)	U.I.
Phosphatidylcholine											
Control	1.0	(11)	16.6 ± 1.6^{a}	35.5 ± 0.9^{a}	6.0 ± 0.5^{a}	25.3 ± 1.7^{a}	53.5 ± 1.3	7.8 ± 0.6^{a}	37.2 ± 1.3^{a}	0.9 ± 0.3	108.6 ± 4^{a}
Control	0.25	(10)	20.0 ± 2.2^{ab}	33.2 ± 1.4^{ab}	8.1 ± 0.7^{b}	19.1 ± 1.6^{b}	56.3 ± 1.5	10.4 ± 1.0^{b}	30.4 ± 2.2^{b}	1.4 ± 0.4	100.0 ± 6^{ab}
Diabetic	1.0	(8)	23.2 ± 2.3^{b}	29.3 ± 1.4^{c}	8.2 ± 1.1^{b}	22.9 ± 2.0^{ab}	56.1 ± 1.7	11.5 ± 1.0^{b}	29.8 ± 2.0^{b}	2.1 ± 0.7	91.9 ± 3^{b}
Diabetic	0.25	(8)	23.6 ± 1.3^{b}	29.9 ± 1.0^{bc}	9.0 ± 0.8^{b}	20.7 ± 1.4^{ab}	56.7 ± 1.3	12.1 ± 1.0^{b}	27.6 ± 1.7^{b}	2.2 ± 0.5	90.8 ± 4^{b}
Phosphatidylethanolamine											
Control	1.0	(10)	10.5 ± 0.8^{a}	31.5 ± 2.2	10.8 ± 1.2	17.2 ± 1.6^{a}	44.6 ± 1.8^{a}	12.9 ± 1.4	39.7 ± 1.3^{a}	1.6 ± 0.4	145.5 ± 4^{a}
Control	0.25	(9)	19.5 ± 2.4^{b}	31.9 ± 2.7	9.8 ± 2.7	11.9 ± 1.4^{b}	55.4 ± 2.7^{b}	14.8 ± 2.1	25.6 ± 2.1^{b}	2.0 ± 0.2	99.8 ± 6^{b}
Diabetic	1.0	(7)	16.2 ± 2.4^{b}	28.4 ± 1.3	11.1 ± 0.7	16.0 ± 1.8^{ab}	48.7 ± 2.0^{ab}	14.9 ± 1.2	32.4 ± 3.3^{c}	1.7 ± 0.5	121.6 ± 9^{c}
Diabetic	0.25	(7)	19.0 ± 1.8^{b}	32.2 ± 2.3	11.5 ± 1.2	15.4 ± 1.5^{ab}	54.5 ± 0.7^{b}	13.9 ± 1.1	28.1 ± 1.8^{bc}	1.8 ± 0.5	104.6 ± 4^{b}
Phosphatidylinositol											
Control	1.0	(9)	21.4 ± 1.6^{a}	31.0 ± 2.3	10.6 ± 1.2^{a}	7.5 ± 0.8	58.8 ± 1.6^{a}	14.6 ± 1.1^{a}	23.1 ± 1.4^{a}	2.5 ± 0.8	101.7 ± 5^{a}
Control	0.25	(6)	30.5 ± 2.4^{b}	27.8 ± 1.8	15.2 ± 2.0^{b}	6.7 ± 1.1	63.7 ± 2.8^{ab}	20.6 ± 1.7^{b}	13.2 ± 1.4^{b}	2.0 ± 0.4	68.6 ± 6^{bc}
Diabetic	1.0	(6)	26.8 ± 3.8^{b}	31.1 ± 2.2	10.0 ± 2.1^{a}	7.2 ± 0.9	63.6 ± 3.0^{ab}	14.7 ± 2.1^{a}	17.4 ± 2.5^{b}	2.2 ± 0.3	78.0 ± 10^{b}
Diabetic	0.25	(7)	33.0 ± 1.8^{b}	30.0 ± 3.0	10.3 ± 1.1^{a}	8.3 ± 0.8	68.5 ± 1.4^{b}	14.5 ± 1.4^{a}	12.9 ± 1.0^{b}	1.3 ± 0.1	54.6 ± 3^{c}
Phosphatidylserine											
Control	1.0	(10)	16.2 ± 1.8^{a}	32.6 ± 1.4^{a}	8.1 ± 0.7^{a}	15.2 ± 1.1^{a}	52.3 ± 1.6^{a}	11.2 ± 0.8^{a}	32.7 ± 2.0^{a}	2.3 ± 0.4	121.5 ± 7^{a}
Control	0.25	(6)	23.5 ± 3.2^{b}	32.0 ± 1.0^{a}	9.4 ± 0.9^{ab}	11.7 ± 1.3^{b}	59.8 ± 2.4^{b}	12.5 ± 0.7^{ab}	22.5 ± 1.9^{b}	2.9 ± 0.8	94.2 ± 8^{b}
Diabetic	1.0	(5)	18.2 ± 2.9^{ab}	35.0 ± 2.7^{ab}	7.5 ± 0.6^{a}	18.6 ± 0.7^{a}	56.4 ± 2.2^{ab}	11.2 ± 0.8^{a}	29.9 ± 2.5^{a}	2.2 ± 0.6	102.0 ± 10^{ab}
Diabetic	0.25	(6)	21.9 ± 2.3^{ab}	28.2 ± 2.0^{c}	10.4 ± 0.9^{b}	10.9 ± 1.4^{b}	56.3 ± 3.0^{ab}	14.5 ± 0.9^{b}	23.0 ± 1.5^{b}	3.5 ± 0.3	96.0 ± 7^{b}
Sphingomyelin											
Control	1.0	(11)	35.8 ± 1.8	26.0 ± 0.5^{ab}	10.2 ± 2.2	7.0 ± 0.8^{ab}	67.5 ± 2.0	$15.9\pm2.1_{b}$	13.1 ± 1.2^{a}	2.9 ± 0.5	64.4 ± 5^{ab}
Control	0.25	(8)	38.8 ± 3.0	27.4 ± 1.8^{ab}	9.9 ± 1.6^{ab}	4.6 ± 0.5^{b}	72.7 ± 2.1	15.0 ± 1.9^{b}	8.0 ± 0.8^{b}	2.7 ± 0.7	47.6 ± 5^{b}
Diabetic	1.0	(7)	35.5 ± 1.8	29.7 ± 2.6^{a}	6.7 ± 0.6	8.1 ± 1.1^{a}	71.9 ± 2.2	12.8 ± 1.4	13.7 ± 1.1^{a}	2.0 ± 0.3	69.2 ± 9^{a}
Diabetic	0.25	(6)	39.0 ± 3.4	24.4 ± 1.1^{b}	10.6 ± 1.6	6.9 ± 1.1^{ab}	70.4 ± 2.1	14.1 ± 1.6	11.1 ± 1.2^{ab}	3.4 ± 0.5	60.8 ± 5^{ab}

Values are mean ± S.E. (n=10) for control and diabetic animals fed the dietary treatments: P/S, polyunsatuated to saturated fatty acid ratio of 1.0 or 0.25. Only major fatty acids are reported. Abbreviations used: U.I., unsaturated index. Other abbreviations are defined in the legend to Table III-1. Values without a common superscript are significantly different ($p<0.05$). Values illustrating the effect of treatment on membrane phospholipid, $C_{20:4(6)}$, total polyunsaturated fatty acids and P/S ratio are illustrated in Figure III-1.

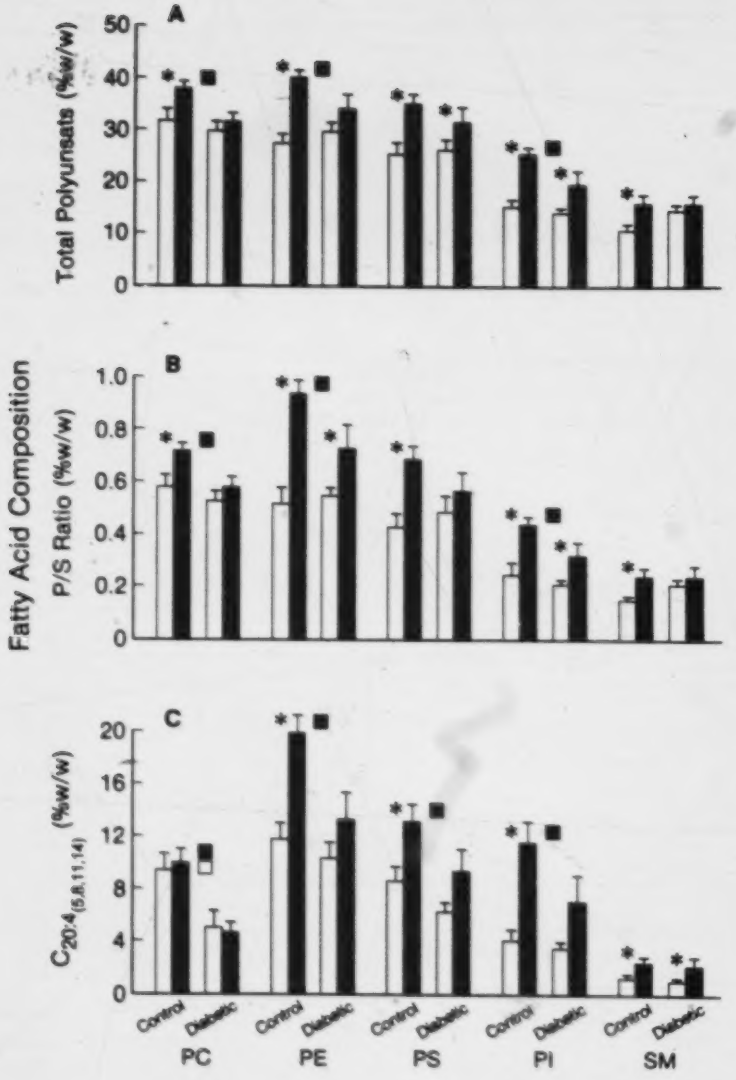

Figure III-1. Effect of diet fat on the polyunsaturated fatty acid composition of adipocyte membrane phospholipids.

Values are group means ± S.E. (n=10). Open bars illustrate the low P/S (P/S=0.25) diet; shaded bars illustrate the high P/S (P/S=1.0) diet. For each phospholipid (*) indicates a significant effect of diet treatment ($p<0.05$); (■) indicates a significant effect of disease state for animals fed the high P/S diet ($p<0.05$); (□) indicates a significant effect of disease state for animals fed the low P/S diet ($p<0.05$). Abbreviations used as follows: total polyunsats, total polyunsaturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin.

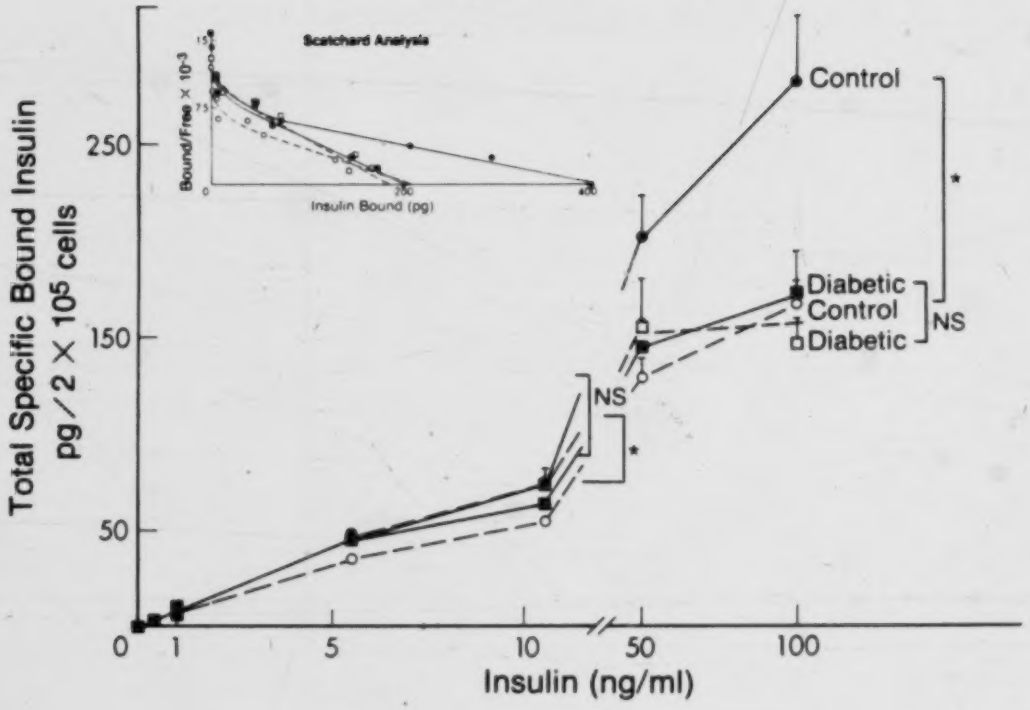

Figure III-2. Relationship between total bound insulin and insulin concentration.

Total insulin bound is expressed as the total specific bound insulin corrected for non-specific bound insulin. The non-specific bound insulin was 5-10% of total insulin bound. Values at each insulin concentration are group means ± S.E. (n=10). The level of significance (*$p<0.01$; **$p<0.001$) is indicated for control animals fed the high P/S diet (●—●); control animal fed the low P/S diet (o—o); diabetic animal fed the high P/S diet (■—■); and diabetic animals fed the low P/S diet (□···□). Scatchard analysis is illustrated for group means (n=10).

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IV. FEEDING A HIGH POLYUNSATURATED FAT DIET IMPROVED GLUCOSE METABOLISM IN DIABETIC ADIPOCYTES [1]

A. INTRODUCTION

Noninsulin dependent diabetes is characterized by a cellular resistance to insulin (Mandarino et al., 1984). Alterations in the binding of insulin to its membrane receptor (Scarlett et al., 1983), glucose transport across the plasma membrane (Scarlett et al., 1983; Ciaraldi et al., 1982) and intracellular glucose metabolism (Arner et al., 1987; Lonnroth et al., 1983; Bolinder et al., 1982) have been reported in adipocytes from individuals with noninsulin dependent diabetes. The streptozotocin diabetic rat also exhibits marked decreases in the ability of insulin to stimulate glucose transport, glucose oxidation, and lipogenesis, despite an apparent increase in insulin binding to its receptor (Karnieli et al., 1981; Kobayashi et al., 1979; Schoenle et al., 1977).

It is well established that the content of dietary fat influences insulin action in adipocytes (Olefsky et al., 1978; Ip et al., 1976). Substituting saturated fat for glucose in the diet has been shown in adipocytes to reduce the number of functional insulin receptors, decrease the activity of the glucose transporter and impair glucose oxidation and incorporation into lipids (Olefsky et al., 1978; Ip et al., 1976). Difficulties exist in extrapolating these results to man as the fat content (greater than 67% of energy) and composition (P/S ratio less than 0.1) in these studies were not representative of diets consumed by man.

Dietary fat-induced alterations in membrane lipid composition have been shown in several tissues to influence the function of membrane proteins, including hormone-mediated functions (Clandinin et al., 1984; Morson & Clandinin, 1985). The insulin receptor (Jarett & Smith, 1974) and the initial step of cellular glucose entry, the glucose transporter (Matthaei et al., 1987), are located in the adipocyte plasma membrane. *In vitro*, using reconstituted vesicles, the insulin receptor (Gould et al., 1982) and glucose transporter (Sandra et al., 1984; Pilch et al., 1980) are sensitive to specific alterations in the content and fatty acyl

[1] A version of this chapter has been submitted for publication. Field CJ, Ryan EA, Thomson ABR & Clandinin MT. (1988) Biochem J

composition of the surrounding membrane phospholipids. Although there is limited *in vivo* work examining the effect of diet-induced membrane alterations on insulin action, replacing a diet containing coconut oil with safflower oil increased adipocyte glucose metabolism (Awad, 1981). As it has been established both in man (Field et al., 1985) and animals (Field et al., 1988) that the type of fat consumed influences the composition of the adipocyte phospholipids, it is logical to hypothesize that diet-induced alterations in membrane composition may influence cellular insulin action.

There is little known about the effect of diet fat composition on insulin action in the diabetic state. Moreover, the diabetic state may alter the effect of diet fat on the fatty acid composition of membranes (Field et al., 1988). In reconstituted membranes, specific phospholipid modifications altered glucose transport and oxidation to a greater extent in cells from control than from diabetic animals (Sandra & Fyler, 1982). The following study was designed to examine the effect of feeding two high fat diets which provided a ratio of polyunsaturated to saturated fatty acids (P/S) analogous to that consumed by the North American population, on insulin-mediated glucose metabolism in diabetic and control animals.

B. MATERIALS AND METHODS

Animals and diets

Forty male Sprague-Dawley rats, 4 weeks of age (140 g) were randomly assigned to one of two groups and fed a semi-purified high fat diet containing 27% (w/w) high protein casein, 38% (w/w) carbohydrate as cornstarch and 20% (w/w) fat. The diet was supplemented with essential nutrients as described in Chapter III. The two diets fed differed in the proportion of safflower and hydrogenated beef tallow to provide a polyunsaturated to saturated fatty acid ratio (P/S) of 0.25 (low P/S) or 1.0 (high P/S). Diets were supplemented with linseed oil to provide a linolenic acid content of 1% of the diet fat. Both diets were cholesterol-free by analysis. The fatty acid analysis of the diets fed is illustrated (Figure IV-1). A complete fatty acid analysis of the diets as determined by gas liquid chromotography

is reported in Chapter I (Table I-1). The experimental protocol was identical to that described in Chapter III.

Each day, for a period of 10 days, an animal from each treatment group was sacrificed by decapitation and serum collected for glucose and insulin determinations. Serum glucose and insulin were determined and epididymal adipocytes isolated as described in Chapter III.

Glucose Oxidation and Incorporation into Lipids

The ability of adipocytes to metabolize glucose was determined by a modification of the method of Rodbell (1964). Freshly isolated cells (200 μl approximately $1.0x10^4$ cells) were incubated in 1 ml for 30 min at 37°C, pH 7.4 in Krebs-Ringer Hepes 2% albumin buffer containing varying levels of insulin (0-25 ng) in 10 ml siliconized vacutainers. One mM D-[^{14}C(U)]-glucose (S.A. 100 μCi/mmole, New England Nuclear, Boston, MA) was added to each tube, and a 500 μl polypropylene microcentrifuge tube was suspended in each tube before capping with rubber serum stoppers. Tubes were incubated for 90 mins in a slow shaking H_2O bath. At the end of the incubation 200 μl methylbenzethonium hydroxide (Sigma Chemicals, St. Louis, MO, U.S.A.) was added to each microcentrifuge tube to trap $^{14}CO_2$ and the reaction was stopped by injecting the cell suspension with 250 μl 2N sulfuric acid. To ensure total $^{14}CO_2$ collection, tubes were slowly shaken for an additional 90 mins and microcentrifuge tubes were removed and placed in a 5 ml acidified (1% v/v acetic acid) Aquasol (New England Nuclear, Boston, MA). For determination of ^{14}C incorporation into lipids, the cell suspensions were extracted according to the method of Dole (1956). The upper hexane layer containing lipids was dried and counted in 5 ml Aquasol. Radioactivity was determined in CO_2 and lipids by counting in a Beckman LS 5801 scintillation counter at an efficiency of approximately 97%. Results were corrected by subtraction of blank reaction tubes (containing cells but no isotope) and the rate of glucose oxidized and incorporated into lipids in 90 min was calculated on a per cell basis and expressed per $2.0x10^5$ cells. Cell numbers were determined by 24 hour fixation at 36°C in 2% osmium tetroxide in 0.05 M collidine (made in

0.15 M saline) as described by Hirsh and Gallian (1968) using a Coulter ZM counter and 400 μm aperture (Coulter-Electronics, Vancouver).

Statistical Analysis

Rates for glucose oxidation and lipogenesis were compared between groups by least-squared analysis of variance procedures for unbalanced data with insulin concentration as the repeated measures variable (Harvey, 1975). Individual basal oxidation and lipogenesis rates were compared by analysis of variance procedures and significant effects of treatment were defined utilizing a Duncan's multiple range test (Steel and Torrie, 1980).

C. RESULTS

The diabetic animals weighed significantly less and had higher serum glucose levels and lower serum insulin levels than control animals (Table III-2). The type of fat fed did not influence body weight or serum glucose and insulin levels in control or diabetic animals.

Glucose Oxidation

Adipocytes from control animals oxidized significantly more glucose than diabetic adipocytes (Figure IV-2). However, feeding diabetic animals the high P/S diet as compared with the low P/S diet significantly increased the amount of glucose oxidized for the seven insulin concentrations tested. Diet did not significantly alter the amount of glucose oxidation in control adipocytes at the insulin concentrations measured. A significantly lower basal oxidation rate (at 0 ng/ml insulin) was found in diabetic animals fed the low P/S diet as compared with diet-matched control animals (10.4±3.0 nmoles and 16.0±3.6 nmoles respectively, $p<0.05$). The basal oxidation rate for cells from high P/S fed diabetic animals did not differ significantly from either control group. At sub-maximum oxidation rates (0-1.0 ng/ml insulin) the amount of glucose oxidized by diabetic animals fed the high P/S diet was greater than observed for diabetic animals fed the low P/S diet ($p<0.05$) and did not differ significantly from cells of either control group. At these insulin levels diabetic adipocytes

from the low P/S fed animals oxidized significantly less glucose than cells from the other groups.

Glucose Incorporation Into Lipids

Feeding a high P/S diet compared with a low P/S diet significantly improved glucose incorporation into lipids by adipocytes from control and diabetic animals (Figure IV-3). Adipocytes from diabetic animals fed the high or low P/S diet synthesized less lipid from glucose than cells from diet-matched control animals. Glucose incorporation into lipids by adipocytes from diabetic animals fed the high P/S diet did not differ significantly from the amount of glucose incorporated by control animals fed the low P/S diet. Basal lipid synthesis (0 ng/ml insulin) was significantly higher in adipocytes from control animals fed the high P/S diet than from either diabetic group. Diet fat did not influence basal rates of lipid synthesis.

D. DISCUSSION

Diet and the diabetic state influenced insulin action in the adipocyte. Regardless of diet treatment, adipocytes from diabetic animals oxidized and incorporated less glucose into lipids, indicating reduced responsiveness to insulin than adipocytes from control animals. Feeding the high P/S diet significantly increased the amount of glucose oxidized by diabetic cells (Figure IV-2) and the amount of glucose incorporated into lipids in both diabetic and control adipocytes. Theoretically, a decrease in insulin responsiveness may be due to post-receptor changes such as alterations in the generation of transmembrane signals, formation of a second messenger or activation of enzymes or membrane carriers by the insulin-receptor complex (Pedersen, 1984).

These results support earlier findings that replacing saturated fat with polyunsaturated fat in the diet (15% w/w) enhanced glucose utilization, measured as oxidation and lipogenesis (Awad, 1981). These increases in insulin stimulated glucose metabolism were associated with increased polyunsaturated fatty acid content of adipose tissue (Awad, 1981). Feeding the high P/S as compared with the low P/S diet in the present study would be expected to significantly

increase the polyunsaturated fatty acid content of the major plasma membrane phospholipids in adipocytes from both diabetic and control animals (Field et al., 1987). Further support for the notion of a membrane mediated effect on insulin action can be inferred from *in vitro* work. For example, pretreating adipocytes with liposomes to modify the phospholipid content and fatty acid composition of the membrane altered cellular glucose oxidation (Sandra et al., 1982). These modifications in membrane lipid composition produced in reconstituted systems are usually greater than those that can be induced by diet. However, in using this *in vitro* system, Sandra et al. (1982) reported a greater membrane mediated effect on glucose oxidation in control cells than those from diabetic cells.

The relationship between insulin binding and the diabetic state is not clear. Previous studies have reported reduced binding (Scarlett et al., 1983) or no change in insulin binding for noninsulin dependent diabetic patients (Arner et al., 1987; Lonnroth et al., 1983; Bolinder et al., 1982). Discrepancies between data available for humans may be partly due to the presence or absence of obesity in these subjects (Lonnroth et al., 1983). Adipocytes from streptozotocin diabetic rats have been reported to bind more insulin than control animals (Kobayashi & Olefsky, 1979). We have found that diet-induced increase in the polyunsaturated fatty acid content of adipocyte plasma membrane phospholipids is associated with enhanced insulin binding in adipocytes from control animals (Field et al., 1988), analogous to that reported for *in vitro* studies (Gould et al., 1982). However, the relationship between insulin receptor binding and insulin action is unclear. This is due to the observation that adipocytes possess spare receptors such that a very large alteration in binding may be necessary to see change in insulin responsiveness (Pedersen, 1984). Receptor alterations are unlikely to explain enhanced insulin action in the diabetic state, because the various diets did not alter insulin binding in adipocytes from diabetic animals (Chapter III).

Glucose transport, being an early step in insulin action and the initial step in cellular glucose metabolism, is a logical site for diet to modify cellular insulin action. *In vitro*, glucose transporter activity can be modulated by specific alterations in the surrounding membrane phospholipids (Sandra et al., 1984; Sandra et al., 1981; Pilch et al., 1980; Melchior & Czech,

1979). Decreased glucose transport rates have been clearly demonstrated in noninsulin dependent diabetic subjects (Scarlett et al., 1983) and streptozotocin diabetic rats (Karneli et al., 1981). Although little work has been done examining the effect of feeding different fats on glucose transport, replacing carbohydrates with saturated fat in the diet is reported to decrease glucose transport in adipocytes (Olefsky & Saekow, 1978). Diet induced alterations in glucose transport are unlikely to provide the sole explanation for changes in glucose metabolism observed, as in the presence of 1.0 mM glucose, in the normal state, glucose transport has not been found to be a rate limiting step for determining intracellular glucose metabolism (Pedersen et al., 1982).

The precise events subsequent to the hormone-receptor interaction, potentiating the metabolic action of insulin, are unknown. However, several lines of evidence support the concept that membrane lipids may be involved in insulin action. Insulin stimulates the generation of a lipid-derived mediator from the plasma membrane that has been reported to stimulate lipogenesis (Jarett & Kieckle, 1984). The release of this mediator is reduced by both the diabetic state and by feeding a high fat diet (Jarett and Kiechle, 1984). Recently a membrane bound phospholipid-dependent protein kinase (protein kinase C) was reported to be involved in the mechanism by which insulin stimulates glucose transport (Christensen et al., 1987). Membrane phosphatidylinositol content, which can be rapidly increased by incubation *in vitro* with insulin (Farese et al., 1982) has been shown to be involved in modulating the activity of protein kinase C (Sweet et al., 1987). The β-subunit of the insulin receptor, embedded in the plasma membrane, contains tyrosine kinase activity that may be reduced in the diabetic state (Comi et al., 1979), perhaps explaining why the coupling between the insulin receptor and activation of insulin effector units have been reported to be altered in diabetic subjects (Mandarino et al., 1984). Phospholipase induced hydrolysis or displacement of membrane phospholipids, which normally mask or shield the receptor, is suggested to be involved in coupling the hormone signal from a regulatory to a catalytic site in the membrane (Sandra et al., 1981).

It is well documented that feeding diets very high in saturated fat decreases adipocyte glucose metabolism, when compared with high carbohydrate diets (Olefsky & Saekow, 1978; Ip et al., 1976). High fat diets also suppress fatty acid synthesis, whereas high polyunsaturated fatty acids increase lipogenesis (Figure IV-3; Nelson et al., 1987). The enhanced maximum response to insulin mediated lipid synthesis by control and diabetic adipocytes and glucose oxidation by diabetic cells from animals fed the high P/S diet, as compared to the low P/S, diet may be due to postmembrane alterations. Diet-induced alterations in the activity of the enzymes of the glycolytic or fatty acid synthesis pathways are logical because some of these enzymes have been demonstrated to be reduced in the diabetic state (Saggerson et al., 1987).

The diets fed in the present study provided dietary P/S ratios similar to the P/S ratios consumed by segments of the North American population. North American Diabetes Associations currently recommend that individuals with diabetes increase the polyunsaturated fat content of their diets (American Diabetes Association, 1987). The physiological implications of the finding that a polyunsaturated diet improves glucose utilization in fat cells is unknown. However, it has been shown that muscle cells, a major glucose consuming organ in man, respond to a high saturated fat diet similarly to adipocytes (Grundleger et al., 1984). A polyunsaturated fat diet similar to that fed in this study has also been reported to improve the clinical condition of the diabetic state, possibly by modifying the form and function of the intestine (Rajotte et al., 1988). Obesity is also characterized by additional defects in cellular insulin action (Pedersen et al., 1982; Lonnroth et al., 1983) and because the majority of individuals with noninsulin dependent diabetes are obese the metabolic benefits of replacing saturated fats in the diet with polyunsaturated fats warrants further investigation. The present results suggest that the nature of dietary fat alters insulin mediated glucose metabolism in diabetic adipocytes and insulin stimulated lipogenesis in both the normal and diabetic states. Replacing dietary saturated fats with polyunsaturated fats tended to normalize lipid synthesis from glucose by cells from diabetic animals. Diet-induced alterations in membrane lipid composition may provide the possible mechanism for the increased insulin responsiveness

observed for epididymal adipocytes from animals fed the high polyunsaturated fat diet.

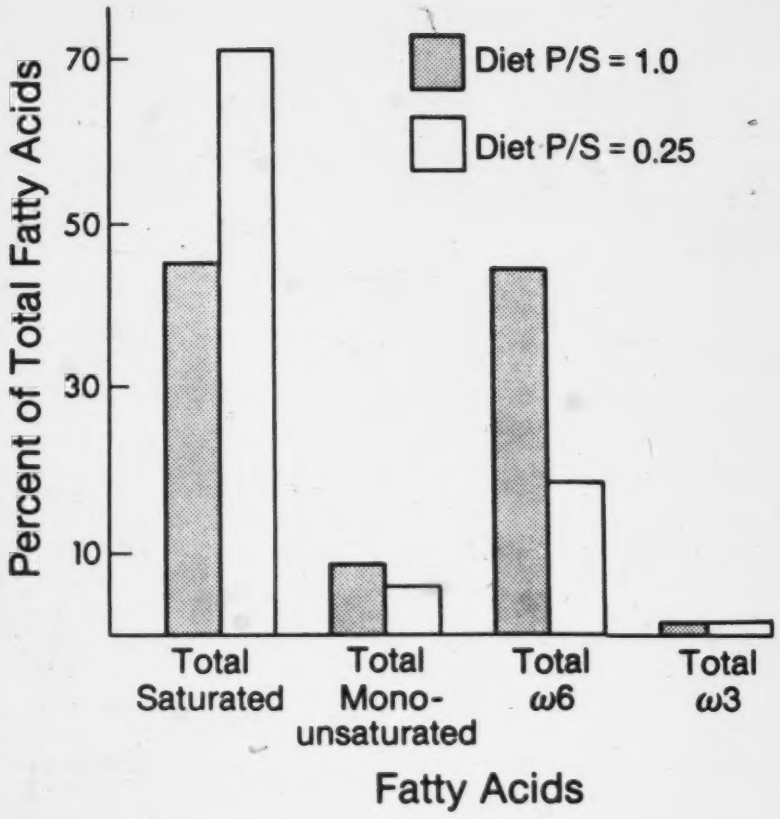

Figure IV-1. Fatty acid composition of diets.

Two high fat diets (20% w/w) differing only in fatty acid composition were fed to rats for 6 weeks. Safflower oil and hydrogenated beef tallow were mixed to provide P/S ratios of 0.25 and 1.0. The diets differed in the amounts of saturated (primarily $C_{18:0}$) and w-6 (primarily $C_{18:2(6)}$) fatty acids.

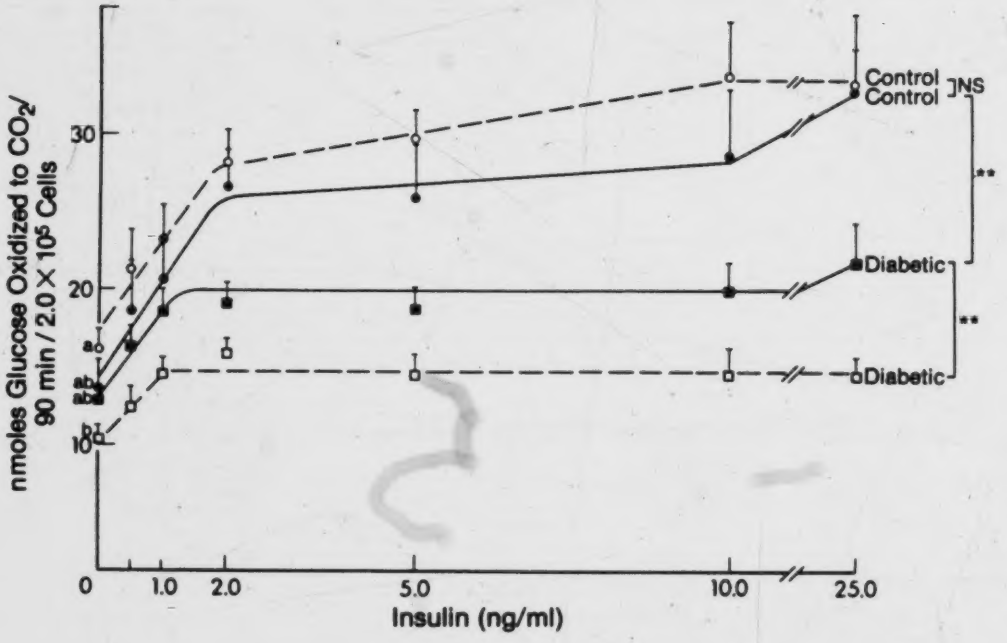

Figure IV-2. Relationships between glucose oxidation to CO_2 and insulin concentration.

Values reported are group means ± S.E. (N=10 animals/group): (●—●) control P/S=1.0; (o·····o) control P/S=0.25; (■—■) diabetic P/S=1.0; (□·····□) diabetic P/S=0.25. Groups were compared to analysis of variance calculated over 7 insulin concentrations tested (**$p<0.01$). Basal oxidation rates were compared by analysis of variance. Values without a common superscript are significantly different ($p<0.05$).

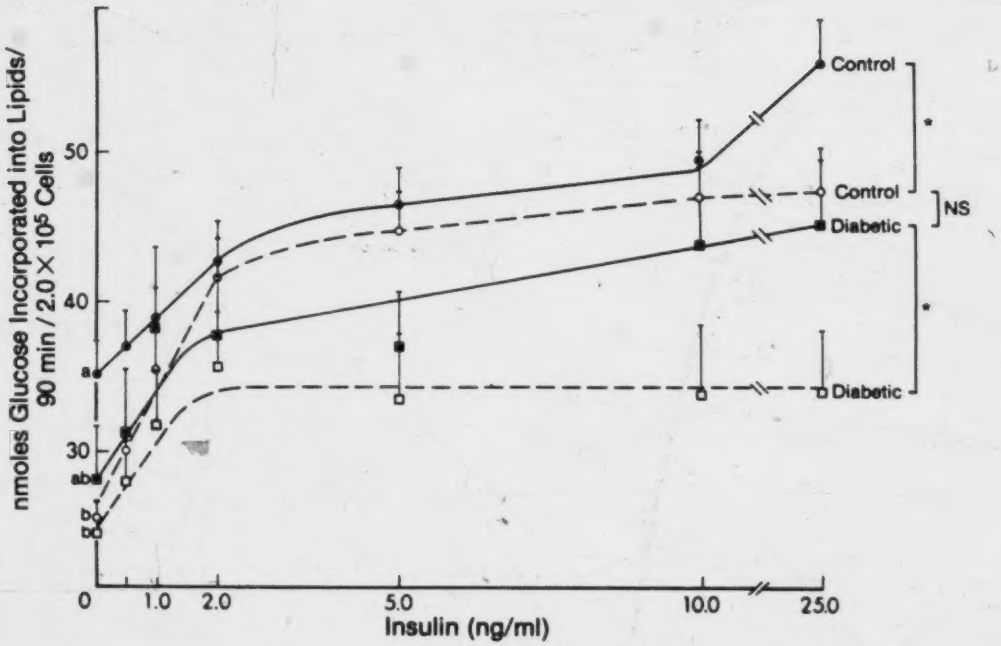

Figure IV-3. Relationship between glucose incorporation into lipid and insulin concentrations.

Values reported are group means ± S.E. (n=10 animals/group): (●—●) control P/S=1.0 (o—o) control P/S=0.25; (■—■) diabetic P/S=1.0; (□···□) diabetic P/S=0.25 Groups were compared by analysis of variance calculated over 7 insulin concentrations tested (*$p<0.05$; **$p<0.01$). Basal lipogenesis rates were compared by analysis of variance. Values without a common superscript are significantly different ($p<0.05$).

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V. IMPROVEMENT OF INSULIN STIMULATED GLUCOSE TRANSPORT IN ADIPOCYTES FROM CONTROL AND DIABETIC ANIMALS BY FEEDING A HIGH POLYUNSATURATED FAT DIET [1]

A. INTRODUCTION

Noninsulin dependent diabetes is characterized by impaired peripheral glucose utilization (Truglia et al., 1985; Scarlett et al., 1983). Glucose transport, an early and important effect of insulin, has been clearly shown to be impaired in both this form of diabetes (Scarlett et al., 1983) and the streptozotocin-induced diabetic rat (Schoenle et al., 1977; Kobayashi et al., 1979; Sandra & Fyles, 1982). In morphological studies both the insulin receptor (Jarett & Smith, 1974) and glucose transporter (Matthaei et al., 1987), are located on the plasma membrane when insulin stimulated. Current understanding of the organization of biological membranes relies on the fluid mosaic model proposed by Singer and Nicholson (1972) in which proteins are embedded to varying degrees in a lipid bilayer. The *in vitro*, activity of both the insulin receptor (Gould et al., 1982; Ginsberg et al., 1981) and glucose transporter (Sandra & Fyler, 1981; Pilch et al., 1980) can be modulated by specific alterations in membrane lipids.

In the animal model, diet fat-induced alterations in membrane composition and function have been clearly illustrated in several tissues. Although it has been well established that the nature of fat fed can alter the fatty acyl composition of the stored and structural lipids in the adipose organ (Field & Clandinin, 1984; Field et al., 1985), the physiological role of these changes on insulin stimulated functions has not been established. There is some indication that dietary fat may influence insulin action because high fat diets, compared to high carbohydrate diets, are reported to induce insulin resistance in adipocytes (Olefsky & Saekow, 1978; Ip et al., 1976; Maegawa et al., 1986). However, the use of unphysiologic levels of highly saturated fats in these studies makes application to the human diet difficult and inconclusive. Insulin binding was found to be enhanced in tumour cells grown in mice fed

[1]A version of this chapter has been submitted for publication. Field CJ, Ryan EA, Thomson ABR & Clandinin MT. (1988) J Biol Chem

diets containing high levels of polyunsaturated fat as compared to cells grown in mice fed a monounsaturated fat (Ginsberg et al., 1982). An improved clinical condition has also been reported in streptozotocin diabetic rats fed a diet containing a high polyunsaturated to saturated fatty acid (P/S) ratio as compared to a low P/S ratio (Rajotte et al., 1988). Recently we demonstrated that feeding a high P/S diet, as compared to a low P/S diet, significantly altered the polyunsaturated fatty acid content of the major phospholipids of the plasma membrane (Field et al., 1987). These diet-induced alterations were associated with improved insulin binding and intracellular glucose metabolism in the normal state and tended to normalize some of the defects in insulin stimulated glucose metabolism that occur in the diabetic state (Field et al., 1987).

The present study was designed to further demonstrate that the composition of diet fat, when consumed at a level and composition analagous to the physiological range of dietary fat intake possible in the North American diet, can alter the membrane mediated processes of insulin binding and glucose transport in the adipocyte. The possible role of alterations in the membrane lipid composition is discussed.

B. MATERIALS AND METHODS

Materials

Streptozotocin, bovine serum albumin (Fraction V), and phloretin were obtained from Sigma Chemicals, St. Louis, MO, U.S.A.) ^{125}I-porcine insulin (specific activity 98 μCi/μg), 3-0-methyl- D-[^{14}C]-glucose (specific activity 55.0 mCi/mmole) and Aquasol were obtained from New England Nuclear, Boston, MA.

Animals and Diets.

Forty weanling male Sprague-Dawley rats (49.2$\pm$1.4 g) were randomly assigned to two diet groups and fed semi-purified high fat diets containing 27% (w/w) high protein casein, 38% (w/w) carbohydrate as cornstarch and 20% (w/w) fat. The diets were

supplemented with essential nutrients as described in Chapter III. The two diets fed differed only in the proportion of safflower oil and hydrogenated beef tallow to provide a polyunsaturated to saturated fatty acid ratio (P/S) of 0.2 (low P/S) or 2.0 (high P/S). The fatty acid analysis by gas liquid chromatography of the diets fed is illustrated in Table V-1.

After feeding the diets for 21 days, diabetes was induced in half of the animals in each diet group by intravenous penile injection of streptozotocin (40 mg/kg body weight) in an acetate buffer (pH 4.5) and the other half with the placebo treatment (acetate buffer). The remaining animals were injected with the placebo treatment (acetate buffer). Animals were continued on their respective diet treatment for an additional 21 days. Only animals with random serum glucose levels greater than 200 mg/dl were considered diabetic.

Each day, for 10 consecutive days, an animal from each treatment group was sacrificed by decapitation and serum collected for glucose and insulin determinations. Serum glucose and insulin were determined as described in Chapter III. Epididymal fat pads were collected and adipocytes isolated by the method of Rodbell (1964), using a modified Krebs-Ringer solution buffered with Hepes (Whitesell & Abumrad, 1985) containing 2% w/v bovine serum albumin and 2 mM pyruvate (pH 7.4).

Freshly isolated adipocytes were incubated for 45 mins at 24°C (pH 7.4) with ^{125}I-porcine insulin in the above buffer described for cell isolation but supplemented with 2mM glucose instead of 2mM pyruvate. Insulin binding was conducted as described by Olefsky and Reaven (1975) in a total volume of 1 ml with approximately 6.0 x 10^4 cells. Optimal steady-state binding conditions at 24°C were achieved for both control and diabetic adipocytes after 45 min of incubation and were maintained for at least 1 additional hour (Appendix, Figure A-1). Binding was terminated as described by Gammeltoft and Gliemann (1973) by rapidly centrifuging 300 μl aliquots from cell suspensions in 500 μl polyethylene microtubes containing 100 μl silicon oil in an MSE centrifuge (John's Scientific, Canada) on high speed (570 g x 1 min). The cells were collected on pipe-cleaners and counted for 2 min in a Packard gamma counter. Counts were corrected for a counting efficiency of 90%. Binding was corrected for non-specific binding by subtracting the amount of insulin bound when cells were

incubated with ^{125}I-insulin in the presence of 25 μg/ml unlabelled insulin and expressed as the amount of insulin bound per 2.0 x 10^5 cells. The nonspecific binding was 6-15% of total binding and did not vary with diet treatment or disease state.

Glucose Transport

The glucose transport activity of fat cells was assessed from the initial uptake of 3-0-methyl-D-[^{14}C]-glucose by modification of the method described by Whitesell & Abumrad (1985). The cell suspension (50 μl) containing approximately 7.5 x 10^3 cells was added to plastic culture tubes containing 10 μl of insulin in varying concentrations and incubated for 30 min at 37°C. Next, 3-0-methyl-D-[^{14}C]-glucose (.4 μCi in 10 μl) was added to each tube, swirled three times and stopped after 5 sec for insulin stimulated transport or 20 sec for basal (no insulin) transport with 300 μl cold phloretin (.3 mM in albumin-free Krebs-Ringer Hepes buffer). Uptake of 3-0-methyl-D-glucose was linear during the time periods measured (Appendix Figures A-3 and A-4). Cells were separated from the buffer by transferring to 500 μl polyethylene microtubes containing 100 μl silicon oil and rapidly centrifuging as described for insulin binding. The tubes were cut through the silicon oil and the cell pellet counted in 5 ml Aquasol in a Beckman LS 5801 scintillation counter at a counting efficiency of approximately 97%. All samples were done in triplicate and corrected for the amount of trapped buffer determined by adding the tracer to cells suspended in 300 μl phloretin. The time between stopping the reaction and separating cells was less than 3 mins. In the first half of the experiment (n=5 animals/group), glucose transport (.5 mM 3-0-methyl-D-glucose) was measured over a range of insulin concentrations (0-1000 ng/ml). In the second half of the experiment (n=5 animals/group), glucose transport was measured over four glucose concentrations (.1, .5, 1.0; 2.0 mM) at 3 insulin levels (0, 1, 1000 ng/ml). Glucose transport was calculated on a per cell basis and expressed as nmoles transported/5 sec. Cell numbers were determined after 24 hour fixation at 36°C in 1% osmium tetroxide in 0.05 M collidine as described in Chapter III.

Statistical Analysis

Rates for glucose transport and total insulin bound were compared between groups by leasted-squared analysis of variance procedures for unbalanced data with insulin and glucose concentrations as repeated measures variables (Harvey, 1975). Values at one insulin concentration and animals' weights and serum levels of glucose and insulin were compared by analysis of variance procedures and significant effects ($p<0.05$) of treatment were further defined utilizing a Duncan's multiple range test (Steel & Torrie, 1980). All data is expressed as mean ± standard error (S.E.).

C. RESULTS

Animals Weights and Serum Glucose and Insulin Levels

After 3 weeks of diet consumption, at the time of induction of diabetes/placebo, animals in the high P/S diet group weighed significantly more than animals in the low P/S diet group (Table V-2). Weight gain during the final 3 weeks of the study was significantly less in diabetic animals as compared to diet-matched controls. Feeding the high P/S diet to both control and diabetic animals improved weight gain as compared to control and diabetic animals fed the low P/S diet. Postprandial serum glucose levels were significantly higher in diabetic animals than control animals. Diabetic animals consuming the high P/S diet had elevated serum glucose levels compared to diabetic animals fed the low P/S diet. Serum insulin levels were significantly lower in diabetic animals than control animals. Control animals fed the high P/S diet had significantly higher serum insulin levels than control animals fed the low P/S diet.

Insulin binding

The total amount of specific insulin bound expressed per 2.0×10^5 adipocytes is presented in Figure V-1. Adipocytes from control animals fed the high P/S diet bound significantly more insulin than cells from control animals fed the low P/S diet ($p<0.001$).

Scatchard analysis (insert to Figure V-1) suggests that the differences are due to a reduction in high affinity binding sites in the low P/S control group. When analyzed across the nine insulin concentrations measured, diet treatment did not influence the amount of insulin bound by cells from diabetic animals. However, at insulin concentration of 1000 ng/ml, adipocytes from diabetic animals fed the low P/S diet bound significantly less ($p<0.05$) insulin than either control group. At all insulin concentrations less than 1000 ng/ml, the amount of insulin bound by adipocytes from diabetic animals was significantly greater than that bound by control cells from animals fed the low P/S diet but did not differ from the high P/S control group.

Glucose transport

For the first half of the experiment (n=5 animals/group), the effect of glucose concentration on glucose transport at three insulin concentrations is presented in Figure V-2. At both basal (Figure V-2C), 1 ng (Figure V-2B) and 1000 ng/ml (Figure V-2C) insulin, across all four glucose concentrations examined, adipocytes from diabetic animals transported significantly less glucose than control adipocytes ($p<0.001$). Diet did not affect the amount of glucose transported by diabetic cells at any insulin concentration. At insulin concentrations of 1000 ng/ml, control cells from animals fed the high P/S diet transported significantly more glucose across the four glucose concentrations than cells from control animals fed the low P/S diet (Figure V-2A; $p<0.001$). For the second half of the experiment (n=5 animals/group) insulin stimulated glucose transport at .5 mM glucose for each group is presented in Figure V-3. Diabetic cells transported less glucose ($p<0.001$) than cells from either control group. At all insulin concentrations tested, adipocytes from control animals fed the high P/S diet transported significantly more glucose than control animals fed the low P/S diet ($p<0.01$). At insulin concentrations less than 25 ng/ml insulin, diet did not affect glucose transport in diabetic animals. However, at the two higher insulin concentrations (100, 1000 ng/ml), glucose transport was improved in cells from animals fed the high P/S diet ($p<0.05$). Although not significant, the tendency for cells from diabetic animals fed the high P/S diet to

transport more glucose at 1000 ng/ml insulin (glucose concentration .5mM) was seen in the first half of the experiment (Figure V-2). The change in insulin stimulated glucose transport expressed as a percent of the amount of glucose transported at insulin concentration of 25 ng/ml for each group is illustrated in the insert for Figure V-3. This figure clearly shows that, for animals fed the high P/S diet, the relative increase in glucose transport at the higher insulin concentrations measured is greater than for animals fed the low P/S diet.

Relationship Between Insulin Binding and Glucose Transport

The mean amount of glucose transported per insulin bound is reduced in diabetic animals (Figure V-4). Diet appeared to influence this relationship. For both control and diabetic animals fed the low P/S diet, mean glucose transport reached a maximum at approximately 350-500 pg insulin bound/2.0 x 10^5 cells. In animals fed the high P/S diet, glucose transport continued to increase as more insulin is bound.

D. DISCUSSION

Feeding a high polyunsaturated fat diet to control animals significantly improved insulin binding to adipocytes. Assuming negative cooperativity, Scatchard analysis suggests this improvement may be associated with an increase in the number of high affinity low capacity binding sites. Dietary fat composition did not significantly affect the amount of insulin bound by cells from diabetic animals when examined over the nine insulin concentrations. However, the amount of insulin bound by adipocytes, from diabetic animals fed the low P/S diet at insulin concentration of 1000 ng/ml, was significantly less than the control groups, suggesting that diet fat composition may have an effect on insulin binding but only at high insulin levels. The enhanced insulin binding to adipocytes from diabetic animals as compared to control animals fed the low P/S diet is consistent with other reports that, despite clear insulin resistance, insulin binding to adipocytes from the streptozotocin diabetic rat compared to the nondiabetic rat is increased (Schoenle et al., 1977; Kobayashi et al., 1979). The magnitude of the enhanced binding has been reported to increase with the severity

of the diabetic state (Kobayashi et al., 1979).

Glucose transport was significantly increased in control animals fed the high P/S as compared to the low P/S diet (Figure V-3) and the magnitude of this difference increased with increases in both glucose and insulin concentrations. Adipocytes from diabetic animals transported less glucose than control cells at all insulin and glucose concentrations. In the streptozotocin diabetic rat, resistance to the stimulatory effect of insulin has been demonstrated to be attributed to decreased translocation of glucose transporters to the plasma membrane as a consequence of a depletion of transporters in the intracelular pool (Kahn et al., 1987). In the present study, feeding the high P/S diet to diabetic animals significantly increased the amount of glucose transported at the higher insulin concentrations (Figure V-3). In both control and diabetic animals, the percent increase in glucose transport after 25 ng/ml insulin was greater in animals fed the high P/S diet as compared to the low P/S diet.

The mechanisms by which diet fat influences insulin receptor function are not clear. However, based on the morphological location of the insulin receptor (Jarett & Smith, 1974) and glucose transporter (Matthaei et al., 1987) within the lipid bilayer of the plasma membrane, several mechanisms can be proposed. Diet-induced alterations in membrane phospholipid composition have been shown in a variety of tissues to influence the function of membrane-associated proteins (Clandinin et al., 1985) including hormone-mediated functions (Neelands & Clandinin, 1983). *In vitro*, both the insulin receptor (Gould et al., 1982; Ginsberg et al., 1981) and glucose transporter (Sandra & Fyler, 1981; Pilch et al., 1980) have been shown to be sensitive to specific alterations in membrane phospholipids. These studies suggest that a more polyunsaturated lipid environment improves insulin binding and insulin stimulated glucose transport. Increasing the polyunsaturated fatty acid composition of the membrane is reported to enhance insulin binding, by increasing the number of binding sites (Gould et al., 1982; Ginsberg et al., 1982) and optimizes glucose transport (Melchior & Czech, 1979). The *in vitro* work has been extended to the intact animals by growing Ehrlich ascites cells in mice fed diets containing a high level of polyunsaturated or monounsaturated fats (Ginsberg et al., 1982). The highly polyunsaturated membrane phospholipids produced

by growing tumour cells in the mice fed the polyunsaturated fat was associated with enhanced insulin binding due to an increased number of receptors (Ginsberg et al., 1982).

The diabetic state is associated with specific alterations in tissue fatty acid compositions that have been suggested to be involved in insulin resistance (Sandra & Fyler, 1982). Reduced levels of monounsaturated and polyunsaturated fatty acids, in particular arachidonic acid ($C_{20:4(6)}$) observed in liver and adipose tissue, have been related to diminished microsomal rates of $\Delta 6$ and $\Delta 9$ fatty acid desaturases (Faas & Carter, 1980; Worchester et al., 1979). Recently it was demonstrated that feeding a high polyunsaturated fat diet attenuated some of the diabetes associated alterations in polyunsaturated fatty acids and was associated with improved intracellular glucose metabolism (Chapters III and IV). Several explanations are evident to help explain the positive effects of membrane alterations induced by feeding the high P/S diet on insulin binding and insulin stimulated glucose transport. Gould et al. (1979) proposed that the accessibility of the insulin receptor for insulin may be controlled by conformational changes in the receptor that require a more polyunsaturated environment. Mobility within a fluid membrane bilayer may be a necessary factor in insulin mediated glucose transport (Melchior & Czech, 1979). Ginsberg et al. (1982) suggested that the receptor may exist in several binding states that are determined by the degree of membrane saturation.

In the present study, feeding the high P/S diet tended to increase the amount of glucose transported per insulin bound in adipocytes in both the normal and diabetic states (Figure V-4). This suggests that diet may influence the coupling between the insulin receptor and the glucose transporter. Although diabetic animals transported less glucose at a given amount of insulin bound, feeding the high P/S diet to diabetic animals tended to normalize this function. The molecular events subsequent to the hormone-receptor interaction causing the metabolic action of insulin on glucose transport are unknown. However, several membrane mediated events have been proposed that due to their contact with membrane lipids might be influenced by diet-induced alterations in the lipid bilayer. Insulin stimulated phosphorylation of the tyrosine residues on the β-subunit of the receptor and the activation of endogenous

insulin receptor kinase are postulated to be critical steps in insulin action (Van Obberghan et al., 1984). A defect in tyrosine kinase activity, associated with the diabetic state (Sinha et al., 1987), has recently been identified as a possible factor inducing insulin resistance (Hari & Roth, 1987). Such a post-binding alteration could help explain the discrepancies observed between insulin binding and glucose transport observed in the streptozotocin model of diabetes. Several plasma membrane generated mediators of insulin action have been proposed (Jarett & Kiechle, 1984; Kelly et al., 1986; Saltiel et al., 1986), all of which have been suggested to contain membrane lipids.

The diets fed in the present study altered some of the clinical characteristics of the animals. The higher body weight and weight gain seen in both control and diabetic animals fed the high P/S diet have also been reported by Rajotte et al. (1988) when feeding high and low polyunsaturated fats. Although food intake was not measured in the present study, these investigators found no apparent differences in food intake between diet groups. Although cell size was not measured in the present study, the relationship between cell size and insulin action is controversial, as both increases (Hood et al., 1984) and decreases (Foley et al., 1986) in insulin mediated glucose transport have been related to cell enlargement. In the present study, serum was collected approximately 2 hours after the end of the dark cycle (i.e. the feeding cycle), representing postprandial rather than fasting levels and thus may not be sensitive measures of the diabetic state. The slightly higher insulin level observed in control animals fed the high P/S diet may relate to the higher weight gain observed in this group or to a reported stimulatory effect of high polyunsaturated fats on pancreatic insulin release (Turk et al., 1987). Practical difficulty exists in producing a uniform diabetic state in all animals. Although serum insulin levels were low in both diabetic groups, blood glucose levels were higher in the high P/S diet group. The fact that the diabetic animals fed the high P/S diet gained more weight cannot be overlooked. Whether this reflects a less severe diabetic state or an improved clinical condition induced by diet remains to be determined.

The physiological implications of improvements in insulin binding and glucose transport by feeding a high polyunsaturated fat diet remain to be tested. However it was

recently demonstrated that feeding a high P/S diet to streptozotocin diabetic rats improved the clinical state as indicated by reduced hemoglobin A1C levels (Rajotte et al., 1988). The present study suggests that diet-induced alterations in the membrane lipid composition of epididymal adipicytes may provide the possible mechanism for the increased insulin binding and glucose transport observed in cells from animals fed a high polyunsaturated diet and that feeding a high polyunsaturated diet may prove beneficial in reducing the insulin resistance of the diabetic state.

Table V.1 Fatty acid composition of diets.

Fatty Acid	Diet	
	High P/S	Low P/S
$C_{14:0}$	0.8	2.5
$C_{14:1}$	0	0.2
$C_{15:0}$	0.1	0.5
$C_{16:0}$	11.0	23.6
$C_{16:1}$	0.2	0.5
$C_{17:0}$	0	0.1
$C_{18:0}$	16.1	53.6
$C_{18:1}$	11.0	4.2
$C_{18:2(6)}$	58.7	12.3
$C_{18:3(6)}$	0.1	0.3
$C_{18:3(3)}$	0.8	1.1
$C_{20:0}$	0.5	0.7
$C_{20:1}$	0.2	0.1
$C_{22:0}$	0.3	0.2
$C_{22:6(3)}$	0.1	0.1
Σpolyunsats	59.9	14.0
Σ(*w*-6)	58.9	12.7
Σ(*w*-3)	1.0	1.3
Σsats	28.2	81.1
Σmonounsats	12.0	5.0
P/S ratio	2.1	0.2

Fatty acid composition of diets fed is expressed as % (w/w) of total fatty acids and was determined by gas liquid chromotography. Abbreviations used: Σpolyunsats, sum of polyunsaturated fatty acis; Σ(*w*-6), sum of (*w*-6)-unsaturated fatty acids; Σ(*w*-3), sum of (*w*-3)-unsaturated fatty acids; Σsats, sum of saturated fatty acids; Σmonounsats, sum of monounsaturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio.

Table V-2. Animal weights, serum glucose and serum insulin levels.

Treatment Group	Diet P/S Ratio	n	Weight (g)	Weight gain (g/day)	Final Serum Glucose (mg/dl)	Final Serum Insulin (μU/ml)
Control	high	9	264±6[a]	8.2±.4[a]	138±17[a]	77±3[a]
Control	low	10	223±4[b]	5.9±.3[b]	126± 2[a]	52±2[b]
Diabetic	high	10	260±5[a]	5.1±.7[b]	381±61[b]	45±5[c]
Diabetic	low	10	234±5[b]	3.9±.2[c]	284±50[c]	43±6[c]

Values are group mean ± S.E. (n=10 animals/group). Weight represents weight after feeding diets for 3 weeks at time of diabetes/placebo treatment. Values without a common superscript are significantly different. 1 ng insulin=25 μU

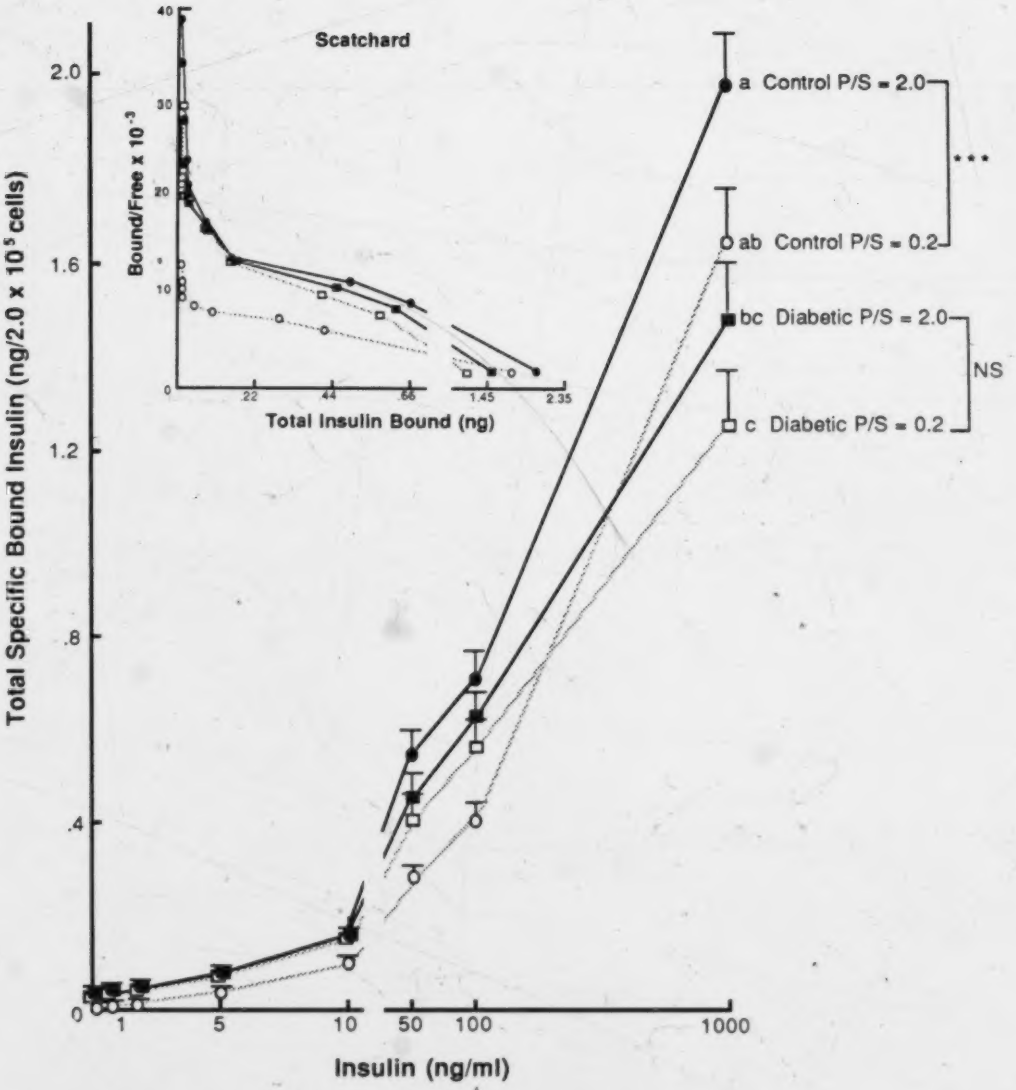

Figure V-1. Total specific bound insulin at 24°C to adipocytes.

Total specific insulin bound at each insulin concentration are group means ± S.E. for 10 control animals fed the high P/S diet (●—●); 9 control animals fed the low P/S diet (o····o); 8 diabetic animals fed the high P/S diet (■—■); and 8 diabetic animals fed the low P/S diet (□—□). The level of significance is indicated (***, $p<0.001$). At 1000 ng/ml insulin values without a common superscript are significantly different ($p<0.05$). Scatchard analysis is illustrated for group means.

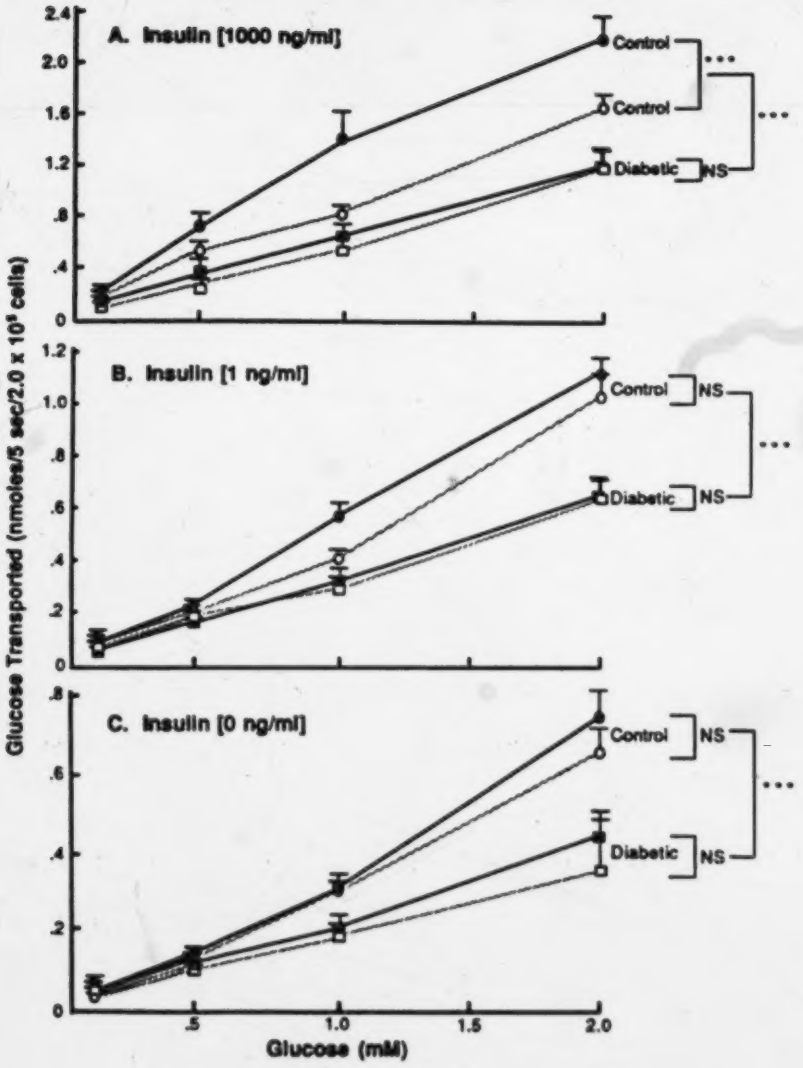

Figure V-2. Effect of glucose concentration on glucose transport.

The effect of glucose concentration on the amount of glucose transported at (A) 1000 ng/ml, (B) 1 ng/ml, (C) 0 ng/ml (basal) insulin concentration for control animals fed the high P/S diet (●—●) control animals fed the low P/S diet (O·····O) diabetic animals fed the high P/S diet (■—■); and diabetic animals fed the low P/S diet (□—□). Values are group means ± S.E. (n=5/group). The level of significance is indicated (***, $p<0.001$).

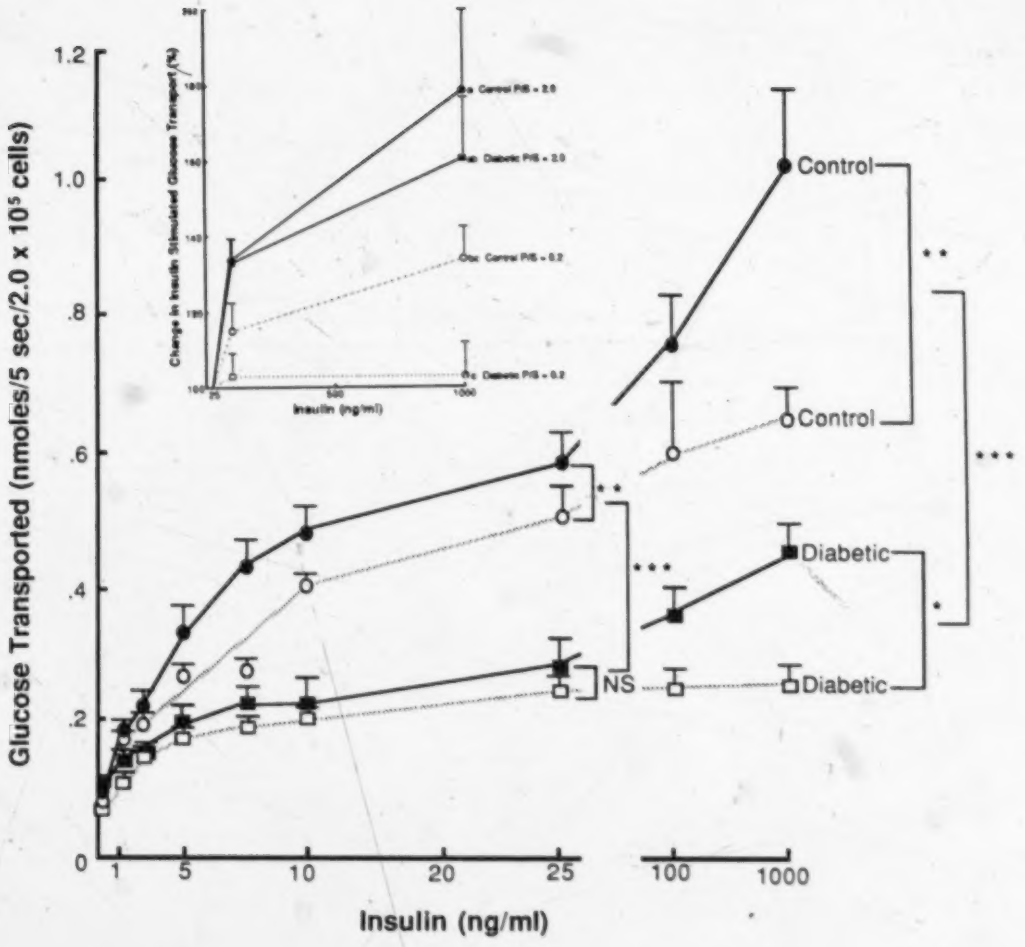

Figure V-3. Insulin stimulated glucose transport.

The amount of glucose transported at .5 mM glucose for control animals fed the high P/S diet (●—●); control animals fed the low P/S diet (o—o); diabetic animals fed the high P/S diet (■—■) diabetic animals fed the low P/S diet (□—□). Values are group means ± S.E. (n=5/group). The level of significance is indicated (*, $p<0.05$), (**, $p<0.01$), (***, $p<0.001$). Statistics were conducted across the lower insulin concentrations (0-25 ng/ml) and the higher insulin concentrations (100, 1000 ng/ml). Using the data illustrated in Figure V-3, the change in insulin stimulated glucose transport expressed as a percent of the amount of glucose transported at 25 ng/ml (100%) for each treatment. Values without a common superscript are significantly different ($p<0.05$).

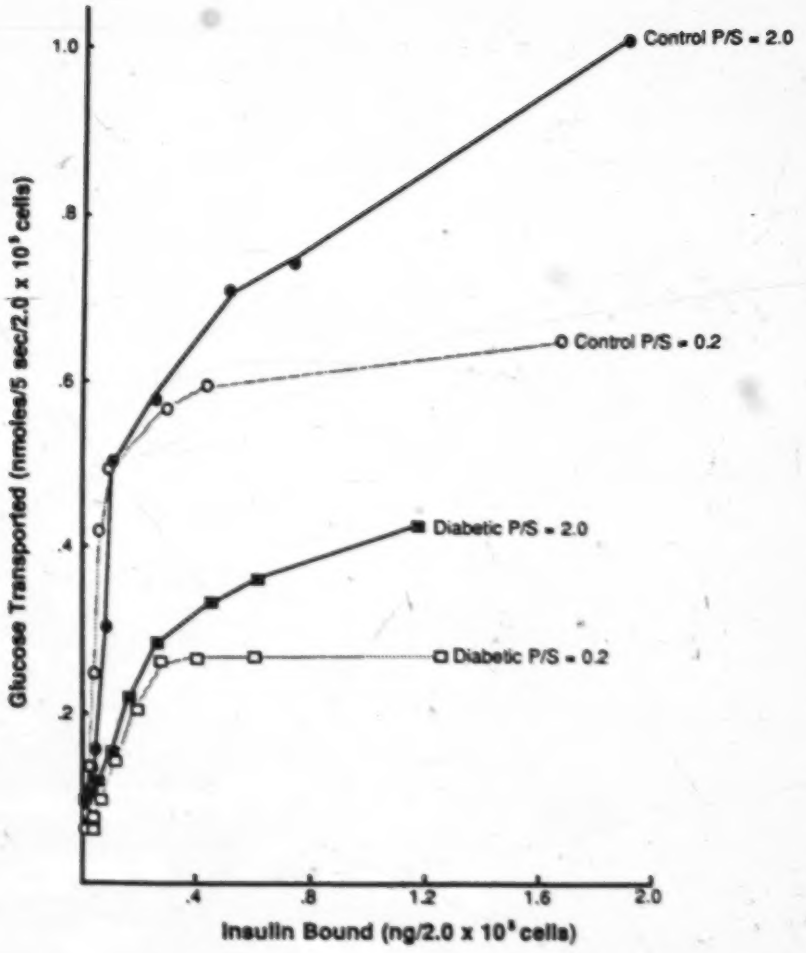

Figure V-4. The relationship between insulin bound and glucose transported.

Values illustrated are group means calculated from the data illustrated in Figure V-1 (amount of insulin bound at each insulin concentration) and Figure V-3 (the amount of glucose transported at each insulin concentration) for control animals fed the high P/S diet (●—●); control animals fed the low P/S diet (o—o); diabetic animals fed the high P/S diet (■—■); diabetic animals fed the low P/S diet (□—□).

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VI. THE EFFECT OF DIETARY FAT COMPOSITION AND THE DIABETIC STATE ON ADIPOCYTE MEMBRANE PHOSPHOLIPIDS [1]

A. INTRODUCTION

Current understanding of the organization of biological membranes relies on the fluid mosaic model, in which proteins are embedded to varying degrees in a lipid bilayer (Singer & Nicolson, 1972). Dietary fat-induced alterations in membrane lipid composition have been shown in several tissues to influence the function of membrane associated proteins (Clandinin et al., 1984; Garg et al., 1988). It is well established in both man and animals that the fatty acid composition of the diet influences the composition of stored lipids in adipose tissue (Field & Clandinin, 1984; Tove & Smith, 1960; Carroll, 1965). In human subjects, a relationship was also found between habitual fatty acid intake and the fatty acyl composition of phosphatidylcholine and phosphatidyl- ethanolamine, two of the major membrane lipids in adipose tissue (Field et al., 1985), thus suggesting that long term dietary fat intake may determine the composition of the major fatty constituents of this organ.

The streptozotocin diabetic state is characterized by a cellular resistance to insulin action (Schoenle et al., 1987; Kobayashi & Olefsky, 1979) and is associated with specific alterations in fatty acid profile of tissues and membranes (Faas & Carter, 1980; Worcester et al., 1979; Brenner, 1968). Lower levels of monounsaturated fatty acids and arachidonic acid ($C_{20:4(6)}$) observed in tissues from diabetic animals may be related to reduced activities of hepatic $\Delta 9$ and $\Delta 6$ desaturases (Faas & Carter, 1980; Worcester et al., 1979; Brenner, 1968). The activities of desaturase enzymes are known to be influenced by both the content and composition of diet fat (Garg et al., 1988; Weekes et al., 1986). The physiological role of these diabetic induced changes and the role of diet in modifying or attenuating them remains to be determined.

The insulin receptor and glucose transporter are located within the lipid bilayer of the plasma membrane (Jarett & Smith, 1976; Matthaei et al., 1987) and are sensitive *in vitro* to

[1] A version of this chapter has been submitted for publication. Field CJ, Ryan EA, Thomson ABR & Clandinin MT. (1988) J Biol Chem

specific alterations in membrane lipids (Ginsberg et al., 1982; Gould et al., 1982; Sandra et al., 1984). Thus, it is logical to hypothesize that diet fat could influence cellular insulin binding and action in both the normal and diabetic states by altering membrane lipid composition. Recently, we demonstrated that replacing a portion of saturated fats with polyunsaturated fats increased the polyunsaturated fatty acyl composition of the major adipocyte membrane phospholipids, attenuated some of the membrane changes associated with the diabetic state and increased insulin binding to control adipocytes (Chapter III). The following study was designed to extend these findings and establish the degree to which transitions in dietary fatty acid intake, achievable by physiological extremes in the human diet, alter the fatty acyl composition of the adipocyte plasma membrane in both normal and diabetic states in the growing animal.

B. MATERIALS AND METHODS

Animals and Diets

Forty weanling male Sprague-Dawley rats (49.2±1.4g) were randomly assigned to two diet groups and fed semi-purified high fat diets as described in Chapter V providing a polyunsaturated to saturated fatty acid ratio (P/S) of 0.2 (low P/S) or 2.0 (high P/S). The major fatty acid composition of the diets fed is illustrated in Figure VI-1. A complete fatty acid analysis of the diets by gas liquid chromatography is provided in Chapter V, Table V-1. The experimental protocol was identical to that described in Chapter V.

Animals were sacrificed over a 10 day period and serum collected for glucose and insulin determinations. Epididymal fat pads were collected and adipocytes isolated as described in Chapter III.

Plasma Membrane Isolation

Plasma membranes were prepared from adipocytes according to the method of Lewis et al. (1979), as described in Chapter III, modified to enable isolation of a larger sample of

plasma membranes in 2 ml. The modification included loading the second pellet, resuspended in 400 μl buffer (0.25M sucrose, 1mM EDTA in 10mM Tris-HCl) at pH 7.4, on a 1600 μl linear gradient containing 32% to 52% (w/v) sucrose, 1mM EDTA in 10mM Tris-HCl buffer pH 7.4. The purity of the plasma membrane fraction was comparable to previous reports (Lewis et al., 1979) and that reported in Appendix I, Table A-1 (data not shown).

Membrane Lipid Analysis

Lipids were extracted from plasma membrane by a modified Folch procedure (Folch et al., 1957) and individual phospholipids were separated on Whatman HP-K thin layer chromatography plates (10 x 10 cm) and compared to appropriate standards as described in Chapter III.

Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol fatty acid methyl esters were prepared using 14% w/v BF_3/methanol reagent (Morrison & Smith, 1964) and separated by automated gas-liquid chromatography as described in Chapter III.

Statistical Analysis

The effect of diet treatment and disease state on membrane phospholipid fatty acid composition and insulin binding were compared by leasted-squared analysis of variance procedures (Harvey, 1975). A Duncan's multiple range test was used to discriminate significant differences between individual treatment groups (Steel & Torrie, 1980). All data reported represent group means ± standard error (S.E.).

C. RESULTS

Animal Weights, Serum Insulin and Glucose

Animals weights, final serum insulin and glucose levels are reported in Chapter V Table V-2. After three weeks of dietary treatment, at the time of induction of diabetes (or

the placebo treatment), animals fed the high P/S diet weighed significantly more than animals in the low P/S diet group ($p<0.05$). Weight gain during the final three weeks of the study was significantly ($p<0.05$) less in diabetic animals as compared to diet-matched control animals. Both control and diabetic animals fed the high P/S diet continued to gain more weight than control and diabetic animals fed the low P/S diet. Serum glucose levels were significantly ($p<0.05$) higher and serum insulin levels significantly ($p<0.05$) lower in the diabetic animals. Although serum insulin levels were similar between diabetic groups, serum glucose levels were higher in diabetic animals consuming the high P/S diet. Serum insulin levels were higher in control animals fed the high P/S diet compared to control animals fed the low P/S diet.

Effect of Diet and the Diabetic State on the Fatty Acyl Composition of Adipocyte Membrane Phospholipids

Diet fat and the diabetic state significantly altered the fatty acyl composition of the major membrane phospholipids of the adipocyte plasma membrane. Fatty acyl composition of each phospholipid as a percent (w/w) is illustrated (Tables VI-1 to VI-4). Significant effects of diet and disease state are also indicated.

Phosphatidylcholine

Feeding a high P/S diet significantly increased the polyunsaturated fatty acid (total polyunsaturated, total *w*-6, P/S ratio and $C_{18:2(6)}$) content while decreasing the monounsaturated fatty acid content (total monounsaturated and $C_{18:1}$) in both normal and diabetic states (Table VI-1). Although the total saturated fatty acid content was not altered by the two diet treatments, feeding the high P/S diet was associated with a higher content of $C_{18:0}$ and lower content of $C_{16:0}$ in membranes from both control and diabetic animals.

The diabetic state was associated with significantly lower levels of monounsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$) and higher levels of polyunsaturated fatty acids (total polyunsaturated, total *w*-6, $C_{18:2(6)}$ and P/S ratio) in phosphatidylcholine. The increase in $C_{18:2(6)}$ and P/S ratio associated with the diabetic state, as compared to control animals,

was greater in diabetic animals fed the high P/S diet and the decrease in $C_{16:1}$ and $C_{18:1}$ was greater for diabetic animals fed the low P/S diet.

Phosphatidylethanolamine

Feeding the high P/S diet significantly increased the polyunsaturated fatty acid (total polyunsaturated, total *w*-6 and $C_{18:2(6)}$) and decreased the monounsaturated fatty acid (total monounsaturated, $C_{16:1}$ and $C_{18:1}$) content in both the normal and diabetic states (Table VI-2). Although the total saturated fatty acid content was not affected by diet, feeding the high P/S diet to both control and diabetic animals was associated with a significant decrease in $C_{16:0}$ and increase in $C_{18:0}$ content. The diabetic state was associated with significantly higher levels of $C_{18:2(6)}$ in animals fed the high P/S diet.

Phosphatidylinositol

Feeding a high P/S diet significantly increased the $C_{18:2(6)}$ content in both control and diabetic animals. Feeding a high P/S diet as compared to the low P/S diet significantly decreased the total monounsaturated fatty acyl content in control animals (Table VI-3). The diabetic state was associated with significantly lower polyunsaturated fatty acid content (total polyunsaturated fatty acids, total *w*-6, $C_{20:4(6)}$ and P/S ratio,) and higher levels of $C_{16:0}$, $C_{16:1}$ and total saturated fatty acids. The increase in total saturated fatty acid and decrease in polyunsaturated fatty acids as compared to control animals was greater for diabetic animals fed the low P/S diet and the increase in $C_{16:1}$ greater for diabetic animals fed the high P/S diet.

Phosphatidylserine

Feeding the high P/S diet significantly decreased the monounsaturated fatty acid (total monounsaturated, $C_{16:1}$ and $C_{18:1}$) and increased the saturated fatty acid (total saturated and $C_{18:0}$) content in both control and diabetic states (Table VI-4). Phosphatidylserine from diabetic membranes was significantly higher in $C_{18:1}$ than diet-matched controls. The effect of the diabetic state on this phospholipid was similar for both diet treatments.

D. DISCUSSION

In both diabetic and control animals, feeding the high P/S diet as compared to the low P/S diet significantly increased the polyunsaturated fatty acid content, in particular the levels of linoleic acid in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, while reducing the monounsaturated fatty acid content in all four of the major adipocyte plasma membrane phospholipids. Although the total saturated fatty acid content was unaltered by diet treatment in all phospholipids except phosphatidylserine, feeding the high P/S diet was associated with higher levels of $C_{18:0}$ and lower levels of $C_{16:0}$. Diet fat composition has been clearly demonstrated in a number of studies to be a major determinant of adipose tissue stored and structural lipids (Field & Clandinin, 1984; Field et al., 1985; Carroll, 1965; Tove & Smith, 1960).

Although generalization can be made about the effect of dietary fats on membrane composition, it is apparent that the influence of diet fat is specific for each of the phospholipid fractions measured. The fatty acyl composition of phosphatidylethanolamine and phosphatidylcholine, the two major membrane phospholipids in most membranes, responded more to diet manipulation than phosphatidylinositol and phosphatidylserine. Although the molecular mechanisms through which dietary fat composition influences the fatty acyl composition of membrane phospholipids are unknown, in addition to altering the available pool of fatty acids for synthesis, dietary fats have been shown to influence: rates of *de novo* synthesis of phospholipids (Hargreaves & Clandinin, 1987a; Takenawa & Nagai, 1982); redistribution of fatty acyl chains via phospholipase (Van den Bosch, 1980) or acyl transferases (Hargreaves & Clandinin, 1987b); and desaturation of membrane phospholipid-linked fatty acids (Garg et al., 1988).

Despite containing a higher content of monounsaturated fatty acids, feeding the high P/S diet reduced the level of monounsaturated fatty acids in all phospholipid fractions measured. A lower level of monounsaturated fatty acids has been reported in other membranes (Garg et al., 1988) and tissues (Weekes et al., 1988) and was associated with reduced hepatic $\Delta 9$ desaturase activity induced by feeding diets high in polyunsaturated fats.

Drug-induced diabetes is associated with considerable alteration in the fatty acid profile of membrane (Faas & Carter, 1980) and tissue (Worcester et al., 1979; Brenner, 1968; Benjamin & Bellhorn, 1964; Friedman et al., 1966) lipids. Reduction in content of monounsaturated fatty acids and $C_{20:4(6)}$ and elevated levels of linoleic acid have been related to diminished rates of hepatic $\Delta 9$ and $\Delta 6$ desaturases induced by the diabetic state (Faas & Carter, 1980; Worcester et al., 1979; Brenner, 1968; Benjamin & Gellhorn, 1964). In the present study, the diabetic state was associated with an elevated content of linoleic acid in phosphatidylcholine and phosphatidylethanolamine and a reduced content of arachidonic acid in phosphatidylinositol. These alterations associated with the diabetic state are consistent with previous reports (Faas & Carter, 1980). Although the levels of $C_{18:1}$ were significantly lower in phosphatidylserine and $C_{16:1}$ lower in phosphatidylcholine, unlike previous reports (Faas & Carter, 1980; Worcester et al., 1979), the diabetic state was not associated with dramatic reduction in monounsaturated fatty acids. In fact, in phosphatidylinositol, diabetic animals exhibited significantly higher levels of $C_{16:1}$. It is conceivable that feeding high fat diets, which provide a physiological content of essential fatty acids, may have prevented this diabetes-induced change in monounsaturated fatty acids; both these factors have been shown to affect $\Delta 9$ desaturase activity in other tissues (Weekes et al., 1986).

The fatty acyl composition of phosphatidylcholine and phosphatidylinositol appeared to be more susceptible to diabetes-induced alterations in membrane lipid composition than phosphatidylethanolamine and phosphatidylserine. This is not unexpected; the pathways for synthesis and degradation as well as the rates of turnover differ for each phospholipid. To some extent, the type of fat fed altered the membrane changes associated with the diabetic state. Compared to control animals fed the same diet, the increase in linoleic acid in phosphatidylcholine and phosphatidylethanolamine observed in membranes from diabetic animals was significantly greater in animals fed the high P/S diet and the decrease in arachidonic acid in phosphatidylinositol was greater in animals fed the low P/S diet. The reason for these differences remains to be determined but may relate to fatty acid availability due to a lower body pool of $C_{20:4(6)}$, or possibly to protective effects induced by feeding a

high P/S diet prior to induction of the diabetic state.

The physiological consequences of plasma membrane alterations remain to be determined. For a variety of other tissues, diet-induced alterations in membrane lipid composition have been clearly shown to influence the function of integral membrane proteins (Clandinin et al., 1984; Hargreaves et al., 1987a; Hargreaves, 1987b; Garg et al., 1988). *In vitro*, the insulin receptor (Ginsberg et al., 1982; Gould et al., 1982) and glucose transporter (Sandra et al., 1984) are sensitive to specific alterations in membrane lipid composition. *In vivo*, increasing the polyunsaturated fatty acid content of membranes by growing tumour cells in mice fed diets containing high levels of polyunsaturated fats, as compared to monounsaturated fats, was associated with enhanced insulin binding (Ginsberg et al., 1982). In Chapter III it was demonstrated that feeding a high P/S diet (1.0), as compared to a low P/S diet (0.25), increased the polyunsaturated fatty acid content of adipocyte membrane phospholipids and enhanced insulin binding. It has been suggested that membrane alterations may influence insulin binding and action by: controlling conformational changes in the receptor (Gould et al., 1979); altering mobility within the lipid bilayer (Melchior & Czech, 1979); or by altering the structure of the receptor (Ginsberg et al., 1982). It is also reasonable to hypothesize that membrane alterations may modify the transduction of the signal generated in the plasma membrane by hormone binding. Membrane alterations have been suggested to influence insulin action post-binding by changing the activities of enzymes involved in phosphatidylinositol turnover (Takenawa & Nagai, 1982) and altering the amount of a plasma membrane mediator of insulin action generated in response to hormone binding (Jarett & Kiechle, 1984).

In the present study, diet fat also influenced some of the clinical characteristics of the animals. Feeding a high P/S diet improved weight gain both before and after induction of diabetes. This is consistent with findings of increased weight gain in control and diabetic animals fed high, as compared to low, polyunsaturated fats (Rajotte et al., 1988). Whether the increased weight gain in diabetic animals represents a less severe diabetic state, possibly due to membrane alterations induced by feeding the polyunsaturated diet before the

streptozotocin injection, or to an improved clinical condition, remains to be determined. Blood for serum glucose and insulin was collected 2 hrs after the end of the dark cycle (ie. the feeding cycle). These samples represent postprandial levels and thus may not be a sensitive indicator of diabetic status. The higher insulin levels in control animals fed the high P/S diet as compared to the low P/S diet may simply relate to increased food intake and weight gain but could also be due to reported stimulatory effect of polyunsaturated fats on pancreatic insulin secretion (Turk et al., 1987).

The present study demonstrates that transitions in dietary fat intake, analogous to those consumed by segments of the North American population, alter the fatty acid composition of the major membrane phospholipids of the epididymal adipocyte plasma membrane in both normal and diabetic states. Feeding a high polyunsaturated fat diet to diabetic animals increased the level of $C_{18:2(6)}$ and prevented the decrease in $C_{20:4(6)}$ in some of the membrane phospholipids. Diet-induced alterations in membrane phospholipid composition by feeding a high P/S diet may provide the mechanism for the improved glucose transport and intracellular glucose metabolism observed in both normal and diabetic animals fed the high P/S diet (Chapter V).

Table VI-1. Effect of diet and the diabetic state on the fatty acid composition of phosphatidylcholine.

Diet Treatment	Fatty Acid Composition (% w/w) Control P/S=2.0 (n=9)	Control P/S=0.2 (n=9)	Diabetic P/S=2.0 (n=10)	Diabetic P/S=0.2 (n=10)	Significant Effect Diet	Significant Effect Disease
$C_{16:0}$	14.6 ±.5[a]	17.0 ± .5 [b]	13.1 ± .4[c]	16.0 ±.5[ab]	•	•••
$C_{16:1}$	1.1 ±.2[a]	1.9 ± .2 [b]	.9 ± .1[a]	1.6 ±.1[c]		••
$C_{18:0}$	34.0 ±.5	29.8 ± .8	33.4 ± .7	30.6 ±.8	•••	
$C_{18:1}$	6.7 ±.5[a]	12.1 ± .6[b]	6.2 ± .4[a]	10.3 ±.3[c]	•	•••
$C_{18:2(6)}$	25.3 ±.9[a]	21.1 ± .9[b]	29.0 ±1.1[c]	24.7 ±.5[b]	•••	•••
$C_{20:4(6)}$	13.7 ±.5	12.1 ± .8	12.2 ± .7	11.6 ±.4		
Σsats	49.9 ±.8	49.0 ± .5	48.2 ± .3	48.5 ±.5		
Σmonounsats	8.8 ±.8	14.4 ±1.5	8.3 ± .6	13.5 ±.5	•••	
Σpolyunsats	41.1 ±.7[ab]	35.3 ± .8[c]	43.2 ± .6[a]	38.4 ±.5[b]	•••	•••
Σ(w-6)	40.1 ±.8[a]	34.0 ± .9[b]	42.3 ± .7[c]	37.3 ±.5[d]	•••	••
Σ(w-3)	.94±.6	1.26± .2	.93± .2	1.14±.1		
P/S	.83±.02[a]	.72±0.1[b]	.90± .01[c]	.79±.02[b]	•••	••

Values represent group means ± S.E. for major fatty acids. Significant effects by two way analysis of variance procedures are indicated (•p<0.05, ••p<0.01, •••p<0.001). No significant interaction was identified. Values without a common superscript are significantly different. Abbreviations used; P/S, polyunsaturated to saturated fatty acid ratio; Σsats, total saturated fatty acids; Σmonounsats, total monounsaturated fatty acids; Σpolyunsats, total polyunsaturated fatty acids; Σ(w-6), total (w-6) fatty acids; Σ(w-3), total (w-3) fatty acids.

Table VI-2. Effect of diet and the diabetic state on the fatty acyl composition of phosphatidylethanolamine.

	Fatty Acid Composition (% w/w)					
	Control		Diabetic		Significant Effect	
Diet Treatment	P/S=2.0 (n=10)	P/S=0.2 (n=7)	P/S=2.0 (n=10)	P/S=0.2 (n=10)	Diet	Disease
$C_{16:0}$	9.2 ± .7	11.6 ± .9	9.3 ±1.1	11.8 ± .6	**	
$C_{16:1}$	1.9 ± .3	2.6 ± .4	2.1 ± .3	2.6 ± .2	**	
$C_{18:0}$	29.4 ± .9	25.3 ± .8	29.9 ± .6	25.3 ± .7	**	
$C_{18:1}$	11.0 ± .2	14.7 ±1.3	10.6 ± .2	13.7 ± .8	**	
$C_{18:2(6)}$	18.6 ±1.0[a]	14.3 ± .7[b]	22.0 ±1.5[c]	15.6 ± .8[b]	**	*
$C_{20:4(6)}$	21.7 ± .7	23.7 ±1.7	19.4 ± .8	21.9 ±1.0		
Σsats	41.0 ± .8	39.5 ±1.4	41.4 ± .8	40.2 ± .8		
Σmonounsats	13.6 ± .4	18.1 ±1.1	12.8 ± .5	17.9 ±1.0	**	
Σpolyunsats	46.1 ±1.0	42.3 ±1.7	46.0 ±1.2	41.8 ±1.3	**	
Σ(w-6)	43.9 ±1.2	39.7 ±1.7	44.6 ±1.2	39.5 ±1.3	**	
Σ(w-3)	2.19± .2	2.69± .2	1.27± .3	2.32± .2		
P/S	1.15± .02	1.09± .08	1.12± .05	1.04± .05		

Values represent group means ± S.E. for major fatty acids. Significant effects by two way analysis of variance procedures are indicated (*$p<0.05$, **$p<0.01$). No significant interaction was identified. Values without a common superscript are significantly different. For abbreviations see Table VI-1.

Table VI-3. Effect of diet and the diabetic state on the fatty acyl composition of phosphatidylinostiol.

Diet Treatment	Fatty Acid Composition (% w/w) Control P/S=2.0 (n=9)	Control P/S=0.2 (n=6)	Diabetic P/S=2.0 (n=10)	Diabetic P/S=0.2 (n=8)	Significant Effect Diet	Disease
$C_{16:0}$	10.3 ±1.1	11.2 ±1.3	13.3 ±1.4	15.2 ±1.8		•
$C_{16:1}$	0.9 ± .2[a]	1.4 ± .3[ab]	1.8 ± .3[b]	1.6 ± .3[ab]		•
$C_{18:0}$	41.1 ±1.9	38.5 ±1.6	42.7 ±2.8	40.3 ±1.3		
$C_{18:1}$	5.6 ± .4	5.8 ± .7	6.7 ± .8	6.4 ± .7		
$C_{18:2(6)}$	5.4 ± .4	3.7 ± .3	5.5 ± .4	4.6 ± .7	••	
$C_{20:4(6)}$	27.7 ±1.3[a]	28.7 ±2.3[a]	22.2 ±1.3[b]	20.7 ±2.6[b]		••
zsats	54.9 ± .8[a]	52.8 ±1.1[b]	56.7 ±1.0[ac]	58.7 ±1.6[c]		••
zmonounsats	7.3 ± .6[a]	9.1 ± .9[b]	10.2 ±1.3[ab]	10.4 ±1.0[ab]	•	
zpolyunsats	36.8 ±1.4[ac]	38.3 ±1.7[a]	33.3 ± .8[bc]	30.6 ±2.1[c]		••
z(w-6)	35.0 ±1.3	35.4 ±2.0	30.8 ±1.1	28.6 ±2.3		••
z(w-3)	2.31± .2	2.88± .7	2.46± .3	2.08± .2		
P/S	0.67± .03[ab]	0.73± .04[a]	0.59± .02[b]	0.53± .05[bc]		••

Values represent group means ± S.E. for major fatty acids. Significant effects by two way analysis of variance procedures are indicated (•p<0.05, ••p<0.01). No significant interaction was identified. Values without a common superscript are significantly different. For abbreviations see Table VI-1.

Table VI-4. Effect of diet and the diabetic state on the fatty acyl composition of phosphatidylserine.

Diet Treatment	Fatty Acid Composition (% w/w) Control P/S=2.0 (n=7)	Control P/S=0.2 (n=8)	Diabetic P/S=2.0 (n=10)	Diabetic P/S=0.2 (n=10)	Significant Effect Diet	Significant Effect Disease
$C_{16:0}$	7.0 ± .7	8.2 ± .8	6.7 ± .8	8.0 ± .9		
$C_{16:1}$	0.8 ± .1	1.1 ± .2	0.6 ± .1	1.4 ± .3	•	
$C_{18:0}$	43.2 ±1.6	40.6 ±1.9	46.9 ±1.8	40.3 ±2.2	•	
$C_{18:1}$	6.6 ± .2	8.2 ± .3	5.1 ± .3	7.7 ± .6	••	••
$C_{18:2(6)}$	18.6 ±1.3	16.2 ±1.0	19.6 ±1.6	17.6 ±1.5		
$C_{20:4(6)}$	12.4 ± .6	11.9 ± .8	11.7 ± .8	10.9 ±1.4		
Σsats	52.3 ± .8	50.9 ±1.2	55.8 ±1.5	51.9 ±1.4	•	
Σmonounsats	8.8 ± .5	12.5 ± .9	8.0 ± .7	11.8 ± .9	••	
Σpolyunsats	38.5 ± .4	37.0 ± .8	36.3 ±1.4	36.1 ±1.3		
Σ(w-6)	35.5 ± .8	34.9 ±1.0	35.2 ±1.4	33.3 ±1.5		
Σ(w-3)	3.03± .9	2.6 ± .4	1.2 ± .3	2.8 ± .6		
P/S	.74± .02	.72± .03	.66± .04	.70± .04		

Values represent group means ± S.E. for major fatty acids. Significant effects by two way analysis of variance procedures are indicated (•p<0.05, ••p<0.01). No significant interaction was identified. Values without a common superscript are significantly different. For abbreviations see Table VI-1.

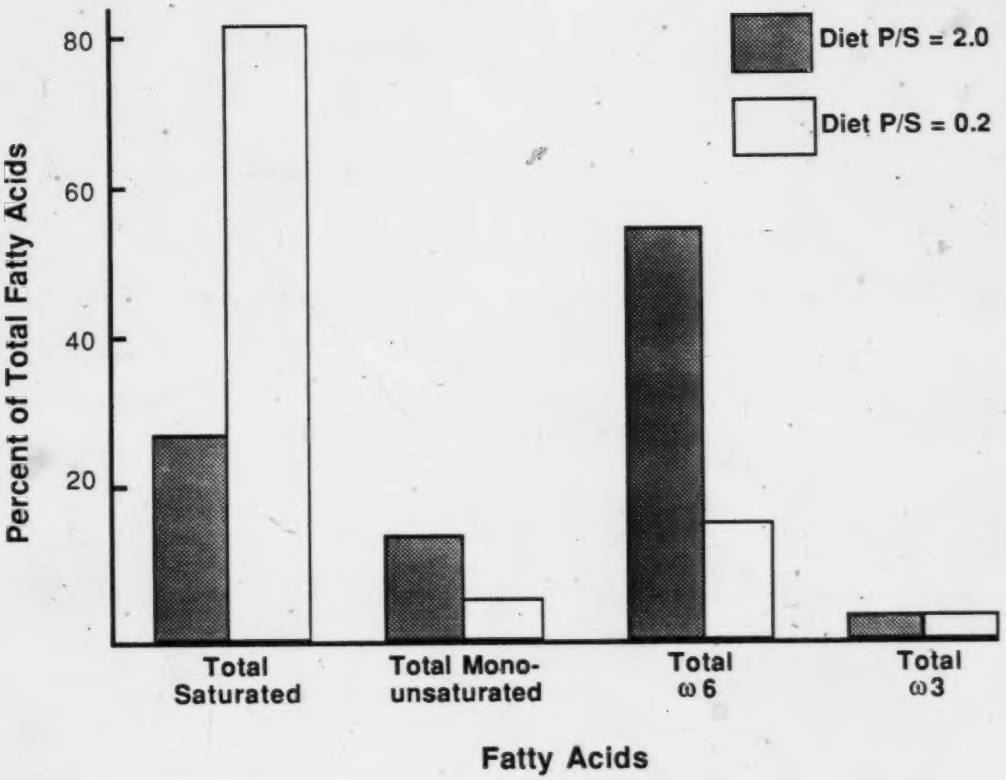

Figure VI-1. Fatty acid composition of diets.

The major fatty acid composition of two high fat diets (20% w/w) fed as determined by gas liquid chromotography. Complete fatty acid analysis is given in Chapter V, Table V-1.

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VII. DIETARY FAT ALTERED THE CONTENT AND COMPOSITION OF ADIPOCYTE MEMBRANE PHOSPHOLIPIDS IN THE NORMAL AND DIABETIC STATE [1]

A. INTRODUCTION

It has been demonstrated in a variety of tissues that the nature of fat consumed alters the composition of membrane phospholipids possibly via: alterations in the rates of *de novo* synthesis of phospholipid (Hargreaves & Clandinin, 1987a); redistribution of fatty acyl chains via phospholipase (Van den Boch, 1980) and acyl transferases (Hargreaves & Clandinin, 1987b); or direct desaturation of membrane phospholipid-linked fatty acids (Garg et al., 1988). The availability of fatty acids at specific sites of synthesis may also be influenced by dietary fat; adipose tissue represents a large pool of available fatty acids, the composition of which to a large extent reflects dietary fatty acid composition (Field & Clandinin, 1984; Field et al., 1985; Carroll, 1965; Awad, 1981). Despite being well established for other membranes, the role of diet fat in determining the composition and content of adipocyte membrane phospholipids has received little attention. Recently, a relationship was established between habitual fat intake and the fatty acyl composition of phosphatidylcholine and phosphatidylethanolamine in human adipose tissue (Field et al., 1985).

The diabetic state, in man (Van-Doormaal et al., 1984) and the streptozotocin diabetic rat (Faas & Carter, 1980; Worcester et al., 1979), is reported to induce considerable alterations in the fatty acid profile of several tissues. Reduced levels of monounsaturated fatty acids and arachidonic acid reported in these studies have been related to decreases observed in rates of hepatic $\Delta 9$ and $\Delta 6$ desaturase enzymes (Faas & Carter, 1980; Worcester et al., 1979; Benjamin & Gellhorn, 1964). However, inconsistencies exist in the reported fatty acid alterations associated with the diabetic state. In particular there are descrepancies in the literature as to the existence of a reduced content of arachidonic acid in diabetic animals or individuals (Faas & Carter, 1980; Worcester et al., 1979; Van Doormaal et al., 1984; Benjamin & Gellhorn, 1964). Possible explanations may be attributed to the variety of

[1] A version of this chapter has been submitted for publication. Field CJ & Clandinin MT. (1988) Diab Nutr Metab

different tissues studied, differences in the nature of dietary fat and the essential fatty acid content of the diet, all of which may affect desaturase activity (Weekes et al., 1986; Garg et al., 1988; Brenner, 1974).

Diet-induced alterations in membrane composition influence the function of membrane associated proteins in a variety of tissues (Clandinin et al., 1985; Hargreaves & Clandinin, 1987b; Garg et al., 1988). In morphological studies the insulin receptor (Jarett & Smith, 1974) and glucose transporter (Matthaei et al., 1987) are located within the plasma membrane. *In vitro*, the insulin receptor (Ginsberg et al., 1981; Gould et al., 1982) and glucose transporter (Sandra & Fyler, 1982; Sandra et al., 1984) are sensitive to specific alterations in membrane lipid composition.

The diabetic state is chatacterized by cellular resistance to insulin action (Sinha et al., 1982; Schoenle et al., 1977; Sandra & Fyler, 1982). Feeding high saturated fat diets compared to low fat high polyunsaturated fat diets induce insulin resistance in animals (Ip et al., 1976; Salans et al., 1981). However, the level and content of fat fed in these studies makes extrapolation to the human diet difficult. The role of dietary fat in determining membrane composition was not established in these studies (Ip et al., 1976; Salans et al., 1981).

In Chapters III and VI, increasing the P/S ratio of diet fat increased the polyunsaturated fatty acyl composition of the adipocyte plasma membrane from both control and diabetic animals. Current dietary recommendations, for both the general population and individuals with diabetes, suggest, in addition to increasing the P/S ratio of the diet, that the total fat content be reduced (American Diabetes Association, 1987; Kisselbach & Schectman, 1988). The following study was designed to clarify the role of the content and composition of dietary fat, when fed in similar proportions as the human diet, on both the composition and content of the major membrane phospholipids of the adipocyte plasma membrane. The role of diet fat in attenuating anticipated changes associated with the diabetic state was also examined. Possible physiological implications on cellular insulin action are discussed.

B. METHODS

Animals and Diets

Sixty weanling male Sprague-Dawley rats (59.7±2.1 g) were randomly assigned to one of four diet groups. Two low fat (10% w/w) and two high fat (20% w/w) diets were fed. The high fat diets were supplemented with essential nutrients as described in Chapter III. To maintain equal essential nutrient density the low fat diets were supplemented with the following per kg: 2.45 g choline, 5.56 g inositol, 2.2 g L-methionine, 8.9 g vitamin mix and 45 g mineral mix. The fat composition of the four diets differed in the proportion of safflower oil and hydrogenated beef tallow, to provide a polyunsaturated to saturated fatty acid (P/S) ratio of 0.2 (low P/S) or 2.0 (high P/S) at each fat level. Linseed oil was added to all four diets to ensure an adequate and constant intake of w-3 fatty acids/g diet. All diets were cholesterol free by analysis. The fatty acid analysis by gas liquid chromatography of the diets is presented (Table VII-1).

The experimental protocol was identical to that described in Chapter V. Animals were sacrificed over a 5 day period by decapitation and serum collected for glucose and insulin determinations. Serum glucose and insulin were analyzed and epididymal adipocytes isolated as described in Chapter III.

Plasma Membrane Extraction and Analysis

Plasma membranes were prepared and lipids extracted as described in Chapter III. Using an aliquot (approximately 3/4 of the extracted lipids), phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine were separated, methyl esters prepared and fatty acids identified by gas liquid chromatography procedures as described in Chapter III.

Membrane Phosphorus Analysis

The remaining extracted lipid was split into two portions. One portion was separated by thin layer chromatography as described in Chapter III and the identified phospholipid scraped into phosphorus-free test tubes. The other portion of lipid was spotted on the silica plates but not developed in the solvent system. This was scraped into phosphorus-free tubes and used to determine total phosphorus. Phosphorus was determined by a modified Bartlett assay for microscale lipid phosphorus analysis (Itoh et al., 1986). The method involved the following in sequence: digestion of the sample in $HClO_4$ at 180°C for 30 mins, air cooling, addition of .880 ml colour-producing agent (.04 ml 5% ammonium molybdate, .80 ml water and .04 ml Fiske and Sabarow reducing reagent) and heating for 10 mins in a boiling water bath. The reaction tubes were cooled and the developed pigment extracted with 1 ml of n-butyl:acetate n-butanol (85:15 v/v). Absorbance was measured at 990 nm with a Perkin-Elmer Lambda 3B spectrophotometer. Phospholipid phosphorus content was calculated as a percent of total phosphorus.

Statistical Analysis

The effect of diet fat content, diet P/S ratio and the diabetic state on the fatty acyl composition of the major saturated, monounsaturated, polyunsaturated and P/S ratio of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were compared by leasted-squared three way analysis of variance procedures (Harvey, 1975). A Duncan's multiple range test was used to discriminate significant differences ($p<0.05$) in animal weights, serum glucose and serum insulin levels (Steel & Torrie, 1980).

C. RESULTS

Animals Weights, Serum Glucose and Serum Insulin

Diabetic animals gained significantly less weight and had significantly higher final serum glucose levels and lower serum insulin levels (Table VII-2). Dietary treatment did not

affect weight gain after streptozotocin/placebo treatment or final serum levels in either control or diabetic animals. After three weeks of feeding the diets, animals in the high fat, low P/S diet group weighed less than the animals fed the other three diets. However, dietary treatment did not alter weight gain in control or diabetic groups during the following three weeks.

Effect of Diet Fat on the Fatty Acyl Composition of Membrane Phospholipids

Phosphatidylcholine

Feeding low fat diets was associated with significantly higher content of monounsaturated fatty acids (total monounsaturated, $C_{16:1}$ and $C_{18:1}$) and a lower polyunsaturated fatty acid content (total polyunsaturated fatty acids, $C_{18:2(6)}$ and P/S ratio) in phosphatidylcholine of membranes from control and diabetic animals (Table VII-3). In animals fed the high P/S diets a higher content of polyunsaturated fatty acids (total polyunsaturated, total *w*-6, $C_{18:2(6)}$ and P/S ratio) and a lower content of monounsaturated fatty acids (total monounsaturated, $C_{16:1}$ and $C_{18:1}$) was observed. In control animals, feeding high P/S diets significantly increased the $C_{20:4(6)}$ content in phosphatidylcholine. Compared to control animals the diabetic state was associated with significantly lower levels of $C_{20:4(6)}$, particularly in membranes from animals fed high P/S diets. Feeding a high fat high P/S diet, compared to a low fat high P/S diet, to diabetic animals reduced the magnitude of the fall in $C_{20:4(6)}$ associated with the diabetic state. In membranes from animals fed high P/S diets a higher level of $C_{18:2(6)}$ was observed and in membranes from diabetic animals fed the high fat diets a reduced level of monounsaturated fatty acids were observed. The total *w*-3 fatty acid content was reduced in diabetic animals.

Phosphatidylethanolamine

Feeding the low fat diets increased the content of monounsaturated fatty acids (total monounsaturated and $C_{18:1}$) and decreased the polyunsaturated fatty acid (total polyunsaturated, $C_{18:2(6)}$ and P/S ratio) content in membranes from control and diabetic animals (Table VII-4). The P/S ratio of the diet did not significantly influence the total

saturated fatty acid content of phosphatidylethanolamine, however, feeding high P/S diets, compared to low P/S diets, increased the $C_{18:0}$ content and reduced the $C_{16:0}$ content in phosphatidylethanolamine. The diabetic state was associated with a reduced content of monounsaturated fatty acids (total monounsaturated and $C_{16:1)}$ and $C_{16:0}$ and an increased content of $C_{18:2(6)}$. In diabetic animals fed high fat diets, the $C_{18:0}$ levels were higher than diet-matched control animals.

Phosphatidylinositol

Feeding high fat diets compared to low fat diets significantly reduced the content of total saturated fatty acids, and increased the content of *w*-3 fatty acids, $C_{20:4(6)}$ and the P/S ratio in the phosphatidylinositol fraction of the adipocyte plasma membrane (Table VII-5). Phosphatidylinositol from membranes of animals fed the high P/S diets, compared to the low P/S diets, was significantly higher in polyunsaturated fatty acids (total polyunsaturated, total *w*-6 and $C_{18:2(6)}$ and P/S ratio) and lower in $C_{16:0}$ and monounsaturated fatty acids (total monounsaturated, $C_{16:1}$ and $C_{18:1}$). The diabetic state influenced the diet effect on phosphatidylinositol composition, but the observed effect related primarily to the fat content of the diet. When low fat diets were fed, the diabetic state was associated with an increased content of $C_{16:0}$ in membranes. In diabetic animals fed high fat diets, compared to control animals, an increased content of $C_{18:0}$ and polyunsaturated fatty acids (total polyunsaturated and *w*-6 fatty acids) and a reduced content of $C_{16:0}$ and $C_{16:1}$ were observed. The diabetic state, compared to diet-matched control animals was associated with a higher content of $C_{18:2(6)}$.

Phosphatidylserine

Feeding a high P/S diet, as compared to a low P/S diet, was associated with higher content of saturated fatty acids (total saturated and $C_{18:0}$) and lower content of monounsaturated fatty acids (total monounsaturated and $C_{18:1}$) in phosphatidylserine (Table VII-6). The content of the essential fatty acid $C_{18:2(6)}$ in phosphatidylserine was significantly increased and the content of *w*-3 fatty acids decreased by feeding high P/S diets to both control and diabetic animals. The fat content in the diet influenced the effect of the

diet P/S on the essential fatty acid content of the membrane. When high fat diets were compared, the high P/S diet was associated with a higher content of $C_{20:4(6)}$ and when low fat diets were compared, the high P/S diet was associated with a higher content of polyunsaturated fatty acids. Lower levels of monounsaturated fatty acids (total monounsaturated and $C_{18:1}$) and higher levels of $C_{18:2(6)}$ were observed in diabetic, compared to diet-matched controls. The level of fat and the P/S ratio of the diet modified the effect of the diabetic state on the fatty acyl composition of phosphatidylserine. A lower level of total saturated fatty acids were observed in diabetic animals fed high fat diets and a lower content of $C_{20:4(6)}$ was observed in membranes from diabetic animals fed the low fat high P/S diets. High P/S diets were associated with lower content of $C_{16:1}$ in phosphatidylserine from diabetic animals, compared to diet-matched control animals.

The Effect of Diet Fat and Diabetes on the Phospholipid Content of the Adipocyte Plasma Membrane

Feeding a high fat diet, as compared to a low fat diet, was associated with a lower content of sphingomyelin, and a higher content of phosphatidylethanolamine in both diabetic and control animals (Table VII-7). In control animals, feeding high fat diets increased the content of phosphatidylinositol in the adipocyte membrane. When high P/S diets compared to low P/S diets, were fed to both treatment groups, a higher content of phosphatidylcholine was observed. A high P/S ratio, compared to a low P/S ratio, as part of a high fat diet was associated with a lower content of phosphatidylethanolamine and a higher content of phosphatidylserine. In membranes from diabetic animals fed the two high fat diets a lower level of phosphatidylethanolamine and a higher level of phosphatidylinositol were observed, compared to the appropriate diet-matched control animals.

D. DISCUSSION

Effect of Diet on Fatty Acid Composition of the Adipocyte Plasma Membrane

In the present study the diabetic state and the content and composition of dietary fat influenced the content and fatty acyl composition of the major phospholipid fractions of the adipocyte plasma membrane. This observation is consistent with previous reports of dietary fat-induced alterations in adipose tissue phospholipids (Field et al., 1985) and in liver plasma (Morson & Clandinin, 1986), microsomal (Garg et al., 1988), synaptosomal (Hargreaves et al., 1987a) and mitochondrial (Innis & Clandinin, 1981) membranes.

As the control of membrane composition is likely multifactorial, the mechanisms by which diet fat alters membrane composition are not clear. However, based on reported effects of diet fat on phospholipid and fatty acid metabolism several mechanisms can be proposed. Diet could increase the availability of certain fatty acids at specific sites of phospholipid metabolism. The essential fatty acid $C_{18:2(6)}$, by definition cannot be synthesized in the body, therefore its presence in phospholipids can be assumed to originate from the diet. In the present study, feeding a high polyunsaturated (P/S) diet compared to a low P/S diet increased the $C_{18:2(6)}$ content in all of the phospholipid fractions examined. The total intake of $C_{18:2(6)}$ is also important as high fat diets, compared to low fat diets, were associated with higher levels of polyunsaturated fatty acids in all the major phospholipid fractions.

The body also has the ability to synthesize and modify fatty acids. It is generally agreed that high fat diets inhibit hepatic lipogenesis (Clarke et al., 1977). Recently, however, it was demonstrated that adipose tissue can continue to be a source of *de novo* fatty acid synthesis in rats during high fat dietary regimes (Nelson et al., 1987). In the present study higher levels of saturated 16 and/or 18 carbon fatty acids, the major end products of endogenous fatty acid synthesis, were observed in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol fractions from animals fed high fat as compared to low fat diets. Polyunsaturated fatty acids, compared to saturated fatty acids, have been reported to both inhibit (Clarke et al., 1977; Romsos & Leveille, 1974) or have no

effect (Nelson et al., 1987) on hepatic and adipose fatty acid synthesis. The higher levels of $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$ observed in most of phospholipid fractions from animals fed low, compared to high, P/S diets support the concept that high polyusaturated fat diets inhibit endogenous fatty acid synthesis or alternatively may simply reflect the greater quantity of these fatty acids provided by the low P/S diet.

Although little work has been done on the effects of diet on adipose tissue desaturation an active $\Delta 9$ desaturase enzyme has been demonstrated in adipose tissue (Wahle & Radcliffe, 1977). In most membrane fractions higher levels of $C_{16:1}$ and $C_{18:1}$ were found in animals fed low fat and/or low P/S diets, supporting the concept that $\Delta 9$ desaturation is regulated by the levels of fat, and $C_{18:0}$ content in the diet (Herodek & Csakvary, 1972). In the present study, diet fat influenced the levels of $C_{20:4(6)}$ in several membrane phospholipids. High fat diets were associated with a lower level of $C_{20:4(6)}$ in phosphatidylserine and higher levels of $C_{20:4(6)}$ in phosphatidylcholine and phosphatidylserine

The Effect of the Diabetic State on the Fatty Acyl Composition of the Adipocyte Plasma Membrane

Alterations in fatty acid composition of tissues and membranes, reflective of decreases in rates of insulin sensitive hepatic $\Delta 6$ and $\Delta 9$ desaturation, have been reported to accompany the diabetic condition (Faas & Carter, 1980; Worcester et al., 1979; Brenner, 1974; Benjamin & Gellhorn, 1964). Consistent with previous reports (Faas & Carter, 1980; Worcester et al., 1979; Brenner, 1974; Van Doormaal et al., 1984), in the present study, the diabetic state was associated with a lower content of monounsaturated fatty acids and a higher content of $C_{18:2(6)}$ and $C_{18:0}$ in most phospholipid fractions. Significantly lower content of $C_{20:4(6)}$, presumably due to an inhibition of $\Delta 6$ desaturation has been reported in some tissues (Faas & Carter, 1980; Benjamin & Gellhorn, 1964), but not all (Worcester et al., 1979; Brenner, 1974), from diabetic animals. Compared to control animals, in the present study, significantly lower levels of $C_{20:4(6)}$ and higher levels of $C_{18:2(6)}$ were observed in the

phosphatidylcholine and phosphatidylserine fractions from diabetic animals, particularly those from animals fed low fat high P/S diets. Although $C_{20:4(6)}$ levels were lower in membranes from diabetic animals, compared to diet-matched controls fed high P/S diets, the levels of $C_{20:4(6)}$ in phosphatidylcholine and phosphatidylserine in the membranes from diabetic animals were still significantly higher than diabetic animals fed low P/S diets. The decreased weight gain and hyperphagia characteristic of diabetic animals is unlikely the cause of these alterations; restricting food intake and weight gain of control animals did not produce the same changes in $\Delta 6$ and $\Delta 9$ desaturation and the polyunsaturated and monounsaturated fatty acid profile (Faas & Carter, 1980). Alternatively, membrane alterations in the diabetic state may be caused by changes in the insulin sensitive pathways of fatty acid biosynthesis, lipolysis or fatty acid oxidation (Kelly et al., 1986; Arner et al., 1983)

Effect of Diet and the Diabetic State on the Phospholipid Content of the Adipocyte Membrane

Each phospholipid class has a characteristic fatty acyl pattern that is likely related to some extent to the different pathways for *de novo* synthesis. Previously it has been demonstrated that the composition of diet fat influences the content of phospholipids in synaptosomal (Foot et al., 1982) and mitochodrial (Robblee & Clandinin, 1984) membranes. Although there is limited information on phospholipid synthesis in adipose tissue, it was recently demonstrated that the pathways for the synthesis of phosphatidylcholine and phosphatidylethanolamine are present in this tissue (Casals et al., 1986). In the present study high fat diets, compared to low fat diets, were associated with alterations in the content of the major phospholipid fractions. The P/S ratio of the diet, particularly when fed as part of a high fat diet, was associated with specific changes in the content of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. The diabetic state was associated with reduced content of phosphatidylethanolamine and an increased content of phosphatidylinositol. However, feeding a low fat diet attenuated the alterations in the phospholipid content associated with the diabetic state.

Physiological Implications of Altered Membrane Composition

The physiological consequences of alterations in adipocyte membrane phospholipids remain to be determined. In several other tissues diet-fat induced alterations in membrane lipid composition have been shown to influence the function of integral membrane proteins (Clandinin et al., 1985; Hargreaves & Clandinin, 1987b; Garg et al., 1988), including hormone mediated function (Morson & Clandinin, 1985). The insulin receptor and glucose transporter *in vitro* (Gould et al., 1982; Ginsberg et al., 1981; Sandra et al., 1984; Pilch et al., 1980) and to some extent *in vivo* (Ginsberg et al., 1982; Chapters III and IV) have been demonstrated to be sensitive to alterations in both the content and composition of surrounding membrane lipids. Lipid containing membrane generated compounds have been identified as possible second messengers to insulin action (Begum et al., 1982; Jarett & Kiechle, 1984; Saltiel et al., 1986). Several of these membrane associated compounds have been shown to be reduced by feeding high fat diets (Takenawa & Nagai, 1982; Begum et al., 1982).

Adipocytes from diabetic patients (Sinha et al., 1987) and streptozotocin diabetic rats (Schoenle et al., 1977) respond poorly to insulin. Alterations in membrane lipid composition have been suggested as a possible mechanism for the impaired insulin response (Sandra & Fyler, 1982). In the present study the diabetic state was associated with lower content of *w*-3 fatty acids in phosphatidylcholine and phosphatidylinositol. Recently, it was demonstrated that replacing a small percentage of *w*-6 fatty acids in the diet with *w*-3 fatty acids prevented the development of insulin resistance associated with high fat diets (Storlien et al., 1987). Although not measured, these researchers hypothesized that diet-induced increases in membrane *w*-3 fatty acids may provide the possible mechanism for improved insulin action (Storlien et al., 1987).

The present study demonstrated that the composition and content of diet fat influence membrane lipids of epididymal adipocytes in both the normal and diabetic states. In most phospholipid fractions; high fat diets were associated with a higher level of polyunsaturated fatty acids, a lower level of monounsaturated fatty acids and an increased membrane content of phosphatidylinositol and phosphatidylethanolamine and reduced content of sphingomyelin;

diets providing a high P/S ratio were associated with higher levels of polyunsaturated fatty acids and, when fed as part of high fat diets, an increased content of phosphatidylcholine and phosphatidylserine and a lower content of phosphatidylethanolamine. The diabetic state was associated with higher levels of $C_{18:2(6)}$ and lower levels of monounsaturated fatty acids in most phospholipids and lower content of $C_{20:4(6)}$ in phosphatidylcholine and a lower content of w-3 fatty acids in phosphatidylcholine and phosphatidylserine. Both the P/S ratio and the fat content of the diet influenced alterations in the phospholipid content of essential fatty acids ($C_{20:4(6)}$ and $C_{18:2(6)}$) associated with the diabetic condition. Diet-fat induced alterations in adipocyte membrane lipid composition may provide a possible mechanism for the improved response to insulin observed in animals fed high P/S diets in Chapters IV and V.

Table VII.1 Fatty acid compostion of diets fed.

Fatty Acid	High Fat (20% w/w) P/S=0.2	High Fat (20% w/w) P/S=2.0	Low Fat (10% w/w) P/S=0.2	Low Fat (10% w/w) P/S=2.0
	(% w/w of total fatty acids)			
$C_{14:0}$	2.8	1.2	3.6	1.2
$C_{14:1}$	.1	.1	.2	.1
$C_{15:0}$	.5	.2	.5	.2
$C_{16:0}$	23.9	13.0	26.2	13.2
$C_{16:1}$	.4	.2	.5	.2
$C_{17:0}$	1.8	.6	1.8	.6
$C_{18:0}$	49.7	16.3	47.0	15.9
$C_{18:1}$	4.3	10.7	6.1	11.2
$C_{18:2(6)}$	13.9	55.9	11.2	54.9
$C_{18:3(6)}$	.2	.1	.2	.1
$C_{18:3(3)}$	1.1	1.1	1.7	1.8
$C_{20:0}$	.1	.4	.6	.4
$C_{20:1}$	0	.1	.1	.1
$C_{22:0}$	.1	0	.1	0
Σpolyunsats	15.9	57.0	13.2	56.8
Σ(*w*-6)	14.3	56.0	11.5	55.0
Σ(*w*-3)	1.1	1.1	1.7	1.9
Σsats	78.8	31.6	77.9	31.4
Σmonounsats	4.8	11.3	8.9	11.8
P/S ratio	0.2	1.8	0.2	1.8

Fatty acid composition of diets were determined by gas liquid chromotography. Abbreviations used Σpolyunsats, sum polyunsaturated fatty acids; Σ(*w*-6), sum of (*w*-6) unsaturated fatty acids; Σ(*w*-3), sum of (*w*-3) unsaturated fatty acids; Σsats, sum of saturated fatty acids; Σmonounsats, sum of monounsaturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio.

Table VII-2. Animal weights, serum glucose and insulin levels.

Diet Treatment	P/S	(n)	Body Weight (g)	Weight Gain (g/day)	Final Serum Levels of Glucose (mg/dl)	Insulin (μU/ml)
Control Animals						
High fat	low	(8)	173± 7[a]	6.4±.7[a]	133± 2[a]	53± 8[a]
High fat	high	(8)	205±10[b]	7.4±.5[a]	138± 4[a]	60± 9[a]
Low fat	low	(8)	203± 5[b]	6.7±.2[a]	137± 6[a]	63±10[a]
Low fat	high	(7)	212±13[b]	7.6±.3[a]	144± 5[a]	69± 6[a]
Diabetic Animals						
High fat	low	(7)	188± 8[a]	3.0±.5[b]	413±68[b]	17± 4[b]
High fat	high	(8)	210± 5[b]	4.0±.4[b]	510±20[b]	16± 5[b]
Low fat	low	(7)	210± 6[b]	4.4±.4[b]	509±59[b]	20± 8[b]
Low fat	high	(8)	212± 5[b]	3.2±.5[b]	505±33[b]	23± 5[b]

Values are group means ± S.E. Body weight is weight 3 weeks after initiation of diet at the time of streptozotocin/placebo treatment. Treatments without a common superscript are significantly different ($p<0.05$). 1 ng insulin = 25 μU

Table VII-3. Effect of diet and the diabetic state on the fatty acyl composition of phosphatidylcholine.

	Composition (% w/w)												
	High fat (20% w/w)				Low fat (10% w/w)								
	P/S=2.0		P/S=0.2		P/S=2.0		P/S=0.2		Significant effect				
Fatty Acid	C	D	C	D	C	D	C	D	F	P/S	D	DF	DxP/S
$C_{16:0}$	16.1±.9	14.2±.8	18.9±.6	17.1±1.1	17.9±.4	18.3±1.7	18.6±.7	23.0±2.3					
$C_{16:1}$	1.0±.2	.6±.1	1.8±.1	1.3±.2	1.2±.1	.8±.2	1.7±.4	2.3±.3	•	•••			
$C_{18:0}$	30.4±.6	32.4±.5	27.4±.8	33.2±2.1	29.2±.7	29.3±.7	28.1±1.3	26.3±.9					
$C_{18:1}$	6.9±.5	5.9±.5	10.9±.3	10.3±.4	6.8±.3	7.9±.8	12.1±1.5	13.3±.8	••	•••			
$C_{18:2(6)}$	23.4±.8	29.4±1.0	19.9±1.1	22.3±1.0	23.8±.8	27.6±1.5	19.1±2.2	17.9±2.0	•	•••	••		•
$C_{20:4(6)}$	15.0±.8	12.4±.8	10.6±.4	11.9±1.2	16.3±.7	10.9±1.1	13.5±.8	11.3±.7		••	••	••	••
Σsats	48.5±.8	48.1±.9	49.1±.9	49.7±1.3	48.8±.3	49.2±1.4	48.8±1.0	51.5±.5					
Σmonoun -sats	9.4±.7	7.3±.7	15.0±.6	12.8±.6	9.3±.4	9.7±1.0	15.8±1.9	17.6±1.0	••	•••	•	•	
Σpolyun -sats	42.0±1.3	44.2±1.6	35.6±.7	37.0±1.4	41.3±.6	41.0±1.8	35.4±1.4	30.6±2.2	•	•••			
Σ(w-6)	40.7±1.2	43.4±1.6	33.9±.6	36.0±1.3	40.2±.7	39.8±1.8	32.4±2.4	29.7±2.2		•••			
Σ(w-3)	1.31±.2	.79±.1	1.75±.6	1.07±.3	1.17±.1	1.21±.1	1.17±.2	.83±.1			••		
P/S	.87±.04	.92±.05	.73±.03	.75±.05	.85±.02	.84±.06	.73±.03	.60±.06	•	•••			

Values given are group means ± S.E. (n=6/group). Abbreviations used; C, Control; D, diabetic; P/S, polyunsaturated/saturated fatty acid ratio; Σsats, sum of saturated fatty acids; Σmonounsats, sum of monounsaturated fatty acids; Σ(w-6); sum of w-6 fatty acids; Σ(w-3), sum of w-3 fatty acids. Significant effects by analysis of variance procedures for; F, level of fat; P/S, P/S ratio; D, diabetic state; and significant interactions are indicated; • $p<0.05$; •• $p<0.01$; ••• $p<0.001$.

Table VII-4. Effect of diet and the diabetic state on the fatty acyl composition of phosphatidylethanolamine.

	Composition (% w/w)												
	High fat (20% w/w)				Low fat (10% w/w)				Significant effect				
	P/S=2.0		P/S=0.2		P/S=2.0		P/S=0.2						
Fatty Acid	C	D	C	D	C	D	C	D	F	P/S	D	FxP/S	DF
$C_{16:0}$	7.7±.4	6.1±.4	11.9±.7	11.8±1.4	9.8±.9	10.8±1.8	13.4±1.0	8.8±1.3		•••	•		
$C_{16:1}$	1.7±.2	1.3±.2	3.4±.4	2.2±.2	2.1±.2	2.0±.4	3.2±.5	2.3±.2		•••	••		
$C_{18:0}$	24.1±.7	27.3±.3	22.0±1.1	24.0±.5	23.4±.7	23.1±.9	20.9±1.0	21.6±.3	••	•••	••	•	
$C_{18:1}$	9.6±.3	10.0±.6	13.0±.6	12.0±.6	10.0±.4	10.7±.7	15.6±.9	15.0±1.7	••	•••			
$C_{18:2(6)}$	16.2±.8	21.8±.9	12.3±.5	12.5±.8	13.7±.6	15.5±1.5	9.2±.5	13.8±2.0	••	•••	•••	•	
$C_{20:4(6)}$	23.2±.8	22.9±.4	24.2±.6	24.4±2.1	26.8±.8	20.1±1.9	24.5±1.2	25.9±2.1					
Σsats	37.4±1.1	38.0±.6	39.0±1.4	39.4±1.5	37.4±1.2	40.9±1.8	38.7±1.4	36.7±1.5					
Σmonoun -sats	14.9±.5	12.8±.6	18.9±1.2	16.5±.6	15.0±.3	15.7±1.3	22.8±1.0	19.9±1.5	••	•••	•		
Σpolyun -sats	47.6±1.0	49.5±.5	42.2±1.0	43.8±1.4	47.0±1.1	43.7±3.0	39.2±1.4	43.7±1.5	•	•••			
Σ(w-6)	45.1±.9	48.5±.4	39.3±.8	41.0±1.9	45.0±.8	40.7±3.0	37.2±1.5	41.7±1.5		•••			
Σ(w-3)	2.75±.3	1.17±.30	2.95±.4	2.82±.6	2.07±.4	2.47±.6	1.97±.2	2.02±.3					
P/S	1.29±.06	1.31±.03	1.09±.05	1.13±.07	1.27±.06	1.09±.1	1.02±.07	1.21±.07		•			

Values are group means ± S.E. (n=6/group). Abbreviations are listed in Table VII-3. Significant effects by analysis of variance procedures for; F, level of fat, P/S, P/S ratio; D, diabetic state; and significant interactions are indirected; • $p<0.05$; •• $p<0.01$; ••• $p<0.001$.

Table VII-5. Effect of diet and the diabetic state on the fatty acyl composition of phosphatidylinositol.

	Composition (% w/w)											
	High fat (20% w/w)				Low fat (10% w/w)				Significant effect			
	P/S=2.0		P/S=0.2		P/S=2.0		P/S=0.2					
Fatty Acid	C	D	C	D	C	D	C	D	F	P/S	D	DF
$C_{16:0}$	13.1±.7	8.5±.7	15.7±.7	13.0±1.6	13.6±1.1	15.5±1.8	15.8±3.0	20.0±1.6	••	••		••
$C_{16:1}$	2.7±.3	1.0±.1	3.2±.4	2.6±.4	2.3±.2	2.2±.2	2.4±.4	2.6±.4		•	•	•
$C_{18:0}$	33.0±.9	39.0±1.2	30.4±.8	35.2±2.2	34.1±1.1	33.0±1.7	33.5±1.8	30.8±1.4		•	•	•
$C_{18:1}$	7.1±.4	7.7±.4	11.0±.5	8.1±1.3	8.0±.8	8.1±.4	8.1±.6	9.6±.5		•		
$C_{18:2(6)}$	5.8±.3	9.4±1.0	4.4±.5	5.3±.6	5.8±.4	6.6±1.0	4.7±.3	4.3±.4		•••	•	
$C_{20:4(6)}$	20.9±1.1	20.4±2.8	21.3±.4	21.9±4.0	25.6±1.3	25.5±1.5	24.4±3.9	20.2±1.3	•			
Σsats	50.3±.9	51.3±.8	50.8±.9	51.8±.6	51.8±.5	52.7±1.2	53.5±2.0	56.3±1.4	••			
Σmonoun -sats	13.6±.6	10.5±.5	16.8±.9	13.2±1.5	12.2±.9	11.9±.4	14.2±1.3	14.4±1.0		••		
Σpolyunsats	35.9±.9	38.2±1.6	32.6±1.4	35.3±1.7	36.6±1.1	35.9±1.4	33.1±2.6	29.1±.8		•••		•
Σ(w-6)	32.4±.7	36.2±1.0	30.1±.9	34.3±2.0	34.5±1.2	35.2±1.2	31.3±3.2	28.1±1.1		••		•
Σ(w-3)	3.49±.5	2.09±.3	2.53±.5	1.23±.3	2.06±.3	.96±.1	1.74±.6	1.78±.3	•		••	
P/S	.72±.03	.75±.04	.65±.04	.68±.04	.71±.02	.69±.04	.63±.07	.52±.02	•	•		

Values are group means ± S.E. (n=6/group). Abbreviations are listed in Table VII-3. Significant effects determined by analysis of variance procedures for; F, level of fat, P/S, dietary P/S ratio; D, diabetic state; and significant interactions are indicated; • $p<0.05$; •• $p<0.01$; ••• $p<0.001$.

Table VII-6. — Effect of diet and the diabetic state on the fatty acyl composition of phosphatidylserine.

	Composition (% w/w)													
	High fat (20% w/w)				Low fat (10% w/w)				Significant effect					
	P/S=2.0		P/S=0.2		P/S=2.0		P/S=0.2							
Fatty Acid	C	D	C	D	C	D	C	D	F	P/S	D	FxP/S	DF	DxP/S
$C_{16:0}$	12.1±1.3	6.4±1.2	12.7±1.0	9.6±1.0	10.0±.7	11.3±2.0	11.8±1.4	9.2±1.4			•			
$C_{16:1}$	2.3±.3	1.0±.1	2.3±.3	3.0±.6	1.9±.1	1.0±.2	2.2±.3	1.6±.2		••	•			•
$C_{18:0}$	38.6±2.3	37.4±1.7	31.8±1.8	35.5±1.6	37.8±.7	39.4±1.3	31.8±1.8	36.4±1.5		•••				
$C_{18:1}$	9.9±.7	7.6±.8	12.1±1.4	10.1±.5	9.4±.2	8.1±.9	14.2±2.3	11.9±.8		•••	•			
$C_{18:2(6)}$	16.6±1.1	23.0±1.7	13.7±1.7	14.1±1.6	17.7±.4	20.4±1.5	13.8±1.8	16.1±1.2		•••	•			
$C_{20:4(6)}$	12.4±.6	13.8±.7	10.0±1.0	13.6±1.5	14.4±.6	11.7±1.1	15.3±2.4	15.5±.9	•			•		
Σsats	52.5±.8	46.7±.6	48.0±.8	49.1±1.2	50.2±.5	53.4±1.9	45.9±2.3	47.7±1.5		•			•	
Σmonoun -sats	13.9±.9	10.6±1.0	18.1±1.8	16.4±1.2	13.8±.3	10.6±1.3	21.1±2.2	16.1±1.0		•••	•••			
Σpolyun -sats	33.7±1.1	42.6±1.3	33.7±2.0	34.5±1.9	36.2±.5	36.2±1.9	33.5±1.3	36.1±1.6		•	•			
Σ(w-6)	32.5±1.2	40.8±1.7	30.7±1.9	32.6±2.2	35.0±.6	34.9±1.8	31.7±1.5	34.0±1.6		•	•			
Σ(w-3)	1.2±.2	1.86±.5	3.0±.3	2.0±.5	1.19±.1	1.25±.2	1.78±.3	2.05±.3		••				
P/S	.64±.03	.92±.04	.70±.05	.71±.06	.72±.02	.69±.06	.74±.05	.76±.06						

Values are group means ± S.E. (n=6/group). Abbreviations are listed in Table VII-3. Significant effects determined by analysis of variance procedures for; F, level of fat, P/S, dietary P/S ratio; D, diabetic state; and significant interactions are indicated; • $p<0.05$; •• $p<0.01$; ••• $p<0.001$.

Table VII-7. Effect of diet fat and the diabetic state on the phospholipid content of the adipocyte plasma membrane.

Phospho-lipid	High fat (20% w/w)				Low fat (10% w/w)				Significant effect				
	P/S=2.0		P/S=0.2		P/S=2.0		P/S=0.2						
	C	D	C	D	C	D	C	D	F	P/S	D	FxP/S	DF
PC	34.2±1.5	39.1±1.6	33.8±2.3	31.4±3.3	37.1±1.9	32.5±2.4	32.6±1.4	31.8±2.1		•			
PE	26.5±1.6	19.6±.6	33.1±3.3	28.1±1.1	18.5±1.3	21.6±1.5	19.9±.7	22.2±1.2	•••	••		•	••
PI	6.0±1.4	8.5±1.1	5.8±.6	11.0±1.6	8.9±1.5	9.2±1.0	12.2±1.1	9.5±.8	•				••
PS	13.0±1.7	15.4±1.0	13.0±1.5	9.9±1.7	14.7±.7	15.0±1.0	15.4±1.2	16.4±1.4	••			••	
SM	19.9±2.1	18.1±1.4	14.2±.7	19.9±1.9	20.9±1.8	22.2±.9	19.4±1.3	20.2±1.0	••				

Values are group means ± S.E. for C, control animals and D, diabetic animals fed the different diets. Abbreviations used; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomylein. Significant effects by analysis of variance procedures for; F, level of fat, P/S, P/S ratio; and significant interactions are indicated; • $p<0.05$; •• $p<0.01$; ••• $p<0.001$.

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VIII. EFFECT OF COMPOSITION AND CONTENT OF DIETARY FAT ON GLUCOSE METABOLISM IN NORMAL AND DIABETIC STATES [1]

A. INTRODUCTION

Studies in both man and animals have demonstrated that replacing dietary carbohydrates with fat induces a state of insulin resistance to cellular glucose metabolism (Olefsky & Saedow, 1978; Salans et al., 1981; Lavau et al., 1979; Maegawa et al., 1986; Susini & Lavau, 1978; Grimditch et al., 1987). The diabetic state both in man (Ciaraldi et al., 1982; Sinha et al., 1987) and the streptozotocin-induced rat (Schoenle et al., 1977; Kobayashi & Olefsky, 1979; Sandra & Fyler, 1982) is characterized by insulin resistance. Reducing the fat content or replacing saturated fats with polyunsaturated fats improves both *in vitro* and *in vivo* insulin action in both noninsulin dependent (Hjollund et al., 1983) and insulin dependent (Pedersen et al., 1982a) diabetic patients and the streptozotocin diabetic rat (Chapters IV, V). Difficulties exist in defining the role of diet fat on insulin action as, by design, human dietary studies simultaneously alter both the fat content and the polyunsaturated/saturated fatty acid ratio. Most animal studies (Olefsky & Saedow, 1978; Lavau et al., 1979; Salans et al., 1981) examining the role of diet fat in diabetes have fed unphysiological dietary fats with respect to both the content and composition of the diet fat component. Thus, the role of fat content and composition on insulin action has not been adequately assessed.

It has been clearly demonstrated in a variety of tissues that the nature of fat consumed alters membrane phospholipid composition and several membrane associated functions (Clandinin et al., 1985; Garg et al., 1988; Morson & Clandinin, 1986). Although it is well established in both man and animals that the nature of fat consumed influences the composition of adipose tissue structural and stored lipids (Field & Clandinin, 1984; Field et al., 1985), it is only recently that the effect of these diet induced alterations on insulin action have received attention. In morphological studies, the insulin receptor (Jarett & Smith, 1974), glucose transporter (Matthaei et al., 1987) and several compounds hypothesized to mediate

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cellular insulin action (Begum et al., 1982; Jarett & Kiechle, 1984; Farese et al., 1982) are located in or originate from the plasma membrane. Because of the intimate contact of these functional components with membrane lipids, it is conceivable that alterations in the composition or physical state of adjacent phospholipids could influence cellular insulin action. Both the insulin receptor (Ginsberg et al., 1981; Grunfeld et al., 1981; Gould et al., 1982) and glucose transporter (Sandra & Fyler, 1982; Pilch et al., 1980; Melchior & Czech, 1979) have been demonstrated to be sensitive to specific *in vitro* alterations in membrane lipid composition. Extension of this work to the intact animal has indicated that insulin binding to tumour cells (Ginsberg et al., 1982) and adipocytes (Chapter III and V) can be altered by changing the fatty acid composition of the diet.

In addition to insulin resistance, the diabetic state is associated with specific alterations in membrane and tissue lipid composition that can only be partially corrected with insulin therapy (Faas & Carter, 1980; Worcester et al., 1977). The role of dietary fat in attenuating metabolic changes in the diabetic state or the physiological implications of these metabolic changes is unclear. In Chapters III, VI and VII, a beneficial role for feeding a high P/S diet was proposed; it was demonstrated to attenuate several changes in the membrane lipid composition associated with the diabetic state.

Current dietary recommendations for both the general population and individuals with diabetes are reducing the total fat content and increasing the P/S ratio of the diet (Kissebach & Schectman, 1988). In Chapter VII, modifying both the content and composition of dietary fat was demonstrated to alter the phospholipid content and the essential and nonessential fatty acid composition of adipocyte plasma membrane phospholipids. The following study was designed to extend these observations to a functional component of the adipocyte by examining the role of diet fat content and composition, when fed in physiological proportions designed to reflect the composition of the human diet, on adipocyte glucose metabolism, measured as glucose transport, glucose oxidation and glucose incorporation into lipids. Both the normal and diabetic states were examined by feeding weanling rats diets differing in fat content (10% w/w and 20% w/w) and composition (P/S=0.2 and P/S=2.0).

B. METHODS

Animals and Diets

Sixty weanling male Sprague-Dawley rats (58.2±1.4g) were randomly assigned to one of three diet groups. Two low fat (10% w/w) and one high fat (20% w/w) semi-purified diets were fed. The high fat diet contained 27% (w/w) casein, 38% (w/w) carbohydrate as cornstarch and 20% (w/w) fat. The high fat and low fat diets were supplemented with essential nutrients as described in Chapters III and VII. The fat content in the diets fed differed in the proportion of safflower oil and hydrogenated beef tallow so as to provide a polyunsaturated to saturated fatty acid ratio (P/S) of 0.2 at the high fat level and both 0.2 (low P/S) and 2.0 (high P/S) in the low fat diets. Linseed oil was added to all three diets to ensure an adequate intake of w-3 fatty acids. The fatty acid analysis by gas liquid chromatography of the diets is illustrated in Table VIII-1.

The experimental protocol was identical to that described in Chapter VI. Each day, for 10 consecutive days, one animal from each treatment group was sacrificed by decapitation and serum was collected for glucose and insulin determinations. Serum glucose and insulin were analyzed and adipocytes isolated from epididymal fat pads as described in Chapter V.

Glucose Transport

The glucose transport activity of isolated adipocytes was assessed from the initial uptake of 3-0-methyl-D-[^{14}C]-glucose (S.A. 55μCi/μmole) corrected to 2.0x10^5 cells as described in Chapter V. Transport was measured over a range of insulin concentrations from 0 ng/ml to 1000 ng/ml at a glucose concentration of 0.5 mM.

Glucose Metabolism

Glucose oxidation and incorporation into lipids, over a range of insulin concentrations (0 ng/ml to 25 ng/ml) was assessed at a glucose concentration of 1 mM using D-[^{14}C(U)]-glucose (S.A. 100 μCi/mmole) as described in Chapter IV. Glucose oxidation and

incorporation into lipids were corrected to $2.0x10^5$ cells as described in Chapter V.

Statistical Analysis

Rates of glucose transport, oxidation and incorporation into lipids were compared between groups by leasted-squared analysis of variance procedures for unbalanced data with insulin concentration as the repeated measures variable (Harvey, 1975). Animal weights, serum insulin and glucose levels were compared by analysis of variance procedures and significant effects of treatment defined utilizing a Duncan's multiple range test (Steel & Torrie, 1980). All data is expressed as mean ± standard error (S.E.).

C. RESULTS

Animal Weight, Serum Glucose and Insulin

After three weeks of dietary treatment, at the time of the diabetes induction by streptozotocin, animals fed the low fat high P/S diet weighed significantly more than the animals fed either the high fat low P/S diet or the low fat low P/S diet (Table VIII-2). Weight gain for diabetic animals was significantly less than observed for control animals. However, animals fed the low fat diets of both high and low P/S ratios gained more weight than animals fed the high fat low P/S diet in both control groups and diabetic groups. Serum glucose levels were significantly higher and serum insulin levels significantly lower for diabetic animals than control animals but did not differ between diet groups.

Glucose Transport

Cells from diabetic animals transported significantly less glucose than control cells (Figure VIII-1). Feeding a low fat high P/S diet significantly increased the rate of glucose transport by control adipocytes at the higher insulin concentrations measured (25 to 1000 ng/ml insulin) as compared to adipocytes from animals fed both the high fat and low fat low P/S diets. However, in the diabetic state, adipocytes from animals fed the high fat low P/S

diet transported significantly more glucose than cells from animals fed either low fat diet.

Glucose Oxidation

Diabetic animals oxidized significantly less glucose than control animals at all insulin concentrations measured ($p<0.001$; Figure VIII-2). Diet treatment did not significantly alter the amount of glucose oxidized by adipocytes from diabetic animals. Adipocytes from control animals fed the high fat, low P/S diet oxidized significantly less glucose than cells from either of the low fat diet fed groups. However, feeding a low fat low P/S diet as compared to a low fat, high P/S diet significantly increased glucose oxidation by control cells.

Lipogenesis

Significantly less glucose was incorporated into lipids by adipocytes from diabetic animals as compared to control animals ($p<0.001$; Figure VIII-3). The rate of glucose incorporation into lipids by adipocytes from control animals fed the high fat low P/S diet was significantly less than control animals fed either of the low fat diets. In control animals, the low fat low P/S diet, as compared to the low fat, high P/S diet, was associated with significantly higher rates of glucose incorporation into lipid. However, in adipocytes from diabetic animals, feeding a low fat, high P/S diet significantly ($p<0.01$) improved glucose incorporation into lipid compared to both low P/S diets. Although improved by feeding a low fat high P/S diet, the amount of glucose incorporated into lipids by diabetic cells was still significantly less ($p<0.05$) than the amount incorporated by control adipocytes exhibiting the lowest rate of lipid synthesis (the control animals fed the high fat low P/S diet).

D. DISCUSSION

Stimulation of hexose transport is an early and important effect of insulin that occurs primarily through translocation of glucose transporters from a large intracellular pool to the plasma membrane (Karnieli et al., 1986; Kahn & Cushman, 1987). The diabetic state, as found in this study and by others (Schoenle et al., 1977; Kobayashi & Olefsky, 1979; Sandra

& Fyler, 1982) is characterized by a cellular resistance to insulin stimulated glucose transport. However, the poor metabolic response to insulin in this diabetic model cannot be explained by reduced insulin binding to membrane receptors (Schoenle et al., 1977; Kobayashi & Olefsky, 1979; Sandra & Fyler, 1982). The lower transport capacity of diabetic cells has been demonstrated to result from a decreased translocation of glucose transporters, as a consequence of a depletion of intracellular pools (Kahn et al., 1985; Karnieli et al., 1981). In the present study, feeding either a high fat low P/S diet or a low fat low P/S diet, compared to a low fat high P/S diet, significantly impaired insulin mediated glucose transport in control animals. This is consistent with previous reports in adipocytes (Salans et al., 1981; Maegawa et al., 1986) and muscle preparations (Maegawa et al., 1986; Grimditch et al., 1987) from animals fed high fat low P/S diets compared to low fat high P/S diets. The present study indicates that the dietary P/S ratio may be more important than the level of dietary fat in determining the rate of glucose transport in the normal state. Similarly, Lavau et al. (1979) did not find an effect of the amount of fat in the diet on glucose transport when two saturated fat diets were compared. In Chapter V, where the identical protocol was followed, the maximal amount of glucose transported by animals fed a high fat high P/S diet (not fed in this experiment) was significantly greater than the amount transported by animals fed the high fat low P/S diet used in this study. As reported in Chapter V, the maximum amount of glucose transported by animals fed a high fat high P/S diet was $1.02 \pm .12$ nmoles/5 sec/2.0×10^5 cells (Figure V-3) and was greater than the amount transported by cells from animals fed the low fat high P/S diet in the present study (Figure VIII-1).

The observation that diabetic animals gained less weight (Table VII-2) suggests they also had smaller fat cells. Although the relationship between cell size and insulin mediated metabolism has been the subject of many studies, the results of these studies have not always been in agreement; both decreases (Foley et al., 1986) and increases (Hood et al., 1980) in insulin mediated glucose metabolism have been reported to accompany increased cell size. In both diabetic and control groups, animals fed the high fat low P/S diet gained significantly less weight. This is consistent with the lower weight gain reported for animals fed a high fat

low P/S diet *ad libitum* compared to a high fat high P/S diet as reported in Chapters V and VI. A pair feeding design to ensure equal weight gain between diet groups would not have been a logical approach to the present study because energy deprivation in rats markedly inhibits insulin mediated glucose metabolism (Olefsky, 1976).

The improved transport rate in adipocytes from control animals fed a high polyunsaturated fat diet may relate to the demonstrated effects of dietary fats on membrane composition and function (Clandinin et al., 1985; Chapters III and VI). A number of studies have demonstrated that *in vitro* both the insulin receptor (Ginsberg et al., 1981; Gould et al., 1982) and glucose transporter (Sandra & Fyler, 1982; Pilch et al., 1980; Melchior & Czech, 1979) are sensitive to specific alterations in membrane lipid content and composition. Insulin binding to adipocytes (Ip et al., 1976; Maegawa et al., 1986; Kolterman et al., 1979) and muscle preparations (Grimditch et al., 1987; Maegawa et al., 1986), possibly due to a reduced number of functional receptors, is reduced in animals fed high fat diets composed mainly of saturated fats. In one study comparing high and low fat diets, no effect was found on insulin binding (Salans et al., 1981) which may have been due to the use of a very high P/S ratio in both diets. In Chapter III, it was demonstrated that increasing the P/S ratio in a high fat diet improved insulin binding to adipocytes from control animals.

The activity of the glucose transporter has been clearly demonstrated *in vitro* to be influenced by the composition of the polar head groups (Sandra & Fyler, 1982; Sandra et al., 1984) and fatty acyl tail composition (Pilch et al., 1980) of surrounding membrane lipids. Optimal transport is reported to require a highly unsaturated bilayer (Melchior & Czech, 1979). From the results of the present study (Figure VIII-1 and VIII-2), dietary preconditioning clearly alters glucose transport but the mechanistic explanation in terms of membrane composition remains to be determined.

Diet fat produced a different effect in adipocytes from diabetic animals compared to control animals; the amount of glucose transported by adipocytes from animals fed either of the low fat diets was significantly less than animals fed the high fat diet. This observation, contrary to that observed in control animals, suggests that the level of fat or carbohydrate in

the diet is important in determining glucose transport. In Chapter V it was found that feeding high fat high P/S diet (not used in the present experiment), as compared to a high fat low P/S diet, further improves glucose transport (Figure V-3). The decreased response in animals fed low fat diets could be related to the use of corn starch, an easily digested carbohydrate demonstrated to have a high glycemic index in man (Jenkins et al., 1980), inducing a hyperglycemic state in these animals. In man, hyperglycemic states are associated with reduced glucose transport (Truglia et al., 1985). Although postprandial values are not sensitive indices of the diabetic condition, serum glucose levels did not differ between groups. However, a greater variation in serum glucose values were observed for animals fed the two low fat diets.

The molecular mechanisms by which a polyunsaturated membrane might improve insulin binding and glucose transport can at this point only be hypothesized. A polyunsaturated membrane environment might: increase the accessibility of the receptor for its ligand (Gould et al., 1982); alter the structure of the receptor (Ginsberg et al., 1981); or change the mobility of the transporter in the membrane (Melchior & Czech, 1979).

The diabetic state is associated with considerable alterations in the fatty acid profile of membranes and tissues (Faas & Carter, 1980; Worcester et al., 1979) which may be attenuated or accelerated by changing the type of fat fed (Chapters IV and VI). Moreover, adipocytes from diabetic animals compared to control cells were found to respond differently to *in vitro* alterations in membrane lipids (Sandra & Fyler, 1982).

In the present study, rates of intracellular glucose metabolism, glucose oxidation, and incorporation into lipids were also influenced by diet fat. High saturated fat diets have been previously demonstrated to impair rates of intracellular glucose metabolism in adipocytes (Chapter IV, Salans et al., 1981; Maegawa et al., 1986; Susini & Lavau, 1978; Olefsky & Saedow, 1978) and muscles (Susini & Lavau, 1978). In the present study, the dietary P/S ratio influenced intracellular glucose metabolism; higher rates of glucose oxidation and glucose incorporation into lipid were observed for control animals fed the low fat low P/S diet compared to the high P/S diet. This effect of P/S ratio is the reverse of what was seen when high fat diets were compared in Chapter IV. Although rates of glucose transport measured at

0.5 mM glucose were lower in adipocytes from animals fed the low fat low P/S diet compared to the high P/S diet (Figure VIII-1), glucose transport at the concentration (1 mM) used to determine intracellular glucose metabolism, is not considered in the normal state to be rate-limiting (Olefsky, 1976).

Regulation of intracellular glucose metabolism by insulin is a multistep process and involves many pathways, making the mechanism(s) for the effect of diet fat on glucose metabolism potentially complex. Although high fat diets have been clearly shown to inhibit hepatic lipogenesis, the effect of fat composition remains controversial (Kelly et al., 1986; Clark et al., 1977). Uncertainty has also existed concerning the role of the adipose organ in fatty acid synthesis, until recently, when it was demonstrated that adipose tissue continues to be an important source of *de novo* fatty acid synthesis in the rat during both high and low fat dietary regimes similar to those used in the present study (Nelson et al., 1987). In adipose tissue, feeding a high saturated fat diet, compared to a low fat diet, was associated with reduced activities of many of the major glycolytic and lipogenic enzymes (Begum et al., 1982; Lavau et al., 1979). The role of dietary P/S ratio in modifying intracellular enzymes of glucose metabolism remains to be established. However, diet induced adaptive alterations in membrane composition could be involved.

It was recently demonstrated that replacing some of the fat in the diet with *w*-3 fatty acids from fish oil improved glucose metabolism and prevented the insulin resistance associated with high fat diets (Storlien et al., 1987). Dietary *w*-3 fatty acids have been shown to have dramatic effects on both the polyunsaturated fatty acyl composition and function of membranes (Garg et al., 1988). In the present study, the dietary level of *w*-3 fatty acids fed was constant, but, the ratio of *w*-3 to *w*-6 fatty acids would be expected to differ as the P/S of the diet fat fed is increased. Whether or not the potential interaction is relevant to the polyunsaturated fatty acid composition of the adipocyte plasma membrane remains to be determined.

Several compounds, generated from the plasma membrane in response to insulin, are known to influence the activity of enzymes involved in glucose metabolism (Jarett & Kiechle,

1984; Begum et al., 1982; Saltiel et al., 1986). Diet may play a role in the modulation of the production of these compounds; the production is reported to be reduced in animals fed diets high in saturated fat (Jarett & Kiechle, 1984; Begum et al., 1982). In Chapters III and VII it was demonstrated that diet fat composition influenced the composition of the major membranes of the adipocyte plasma membrane, including phosphatidylinositol, a phospholipid hypothesized to play an important role in mediating insulin action (Farese et al., 1982). The turnover of this phospholipid has been shown to be sensitive to both polyunsaturated fatty acids (Takenawa & Nagai, 1982) and the lipid composition of the membrane (Irvine, 1982).

The present study clearly demonstrates reduced glucose metabolism in cells from diabetic animals. Streptozotocin-induced diabetes is associated with reduced rates for many enzymes involved in fatty acid and triglyceride synthesis (Saggerson et al., 1987). Dietary treatment in the present study did not alter the reduced oxidative capacity of the diabetic cells, but, feeding a low fat high P/S diet increased glucose incorporation into lipids. It has been reported by others that the rates of glucose oxidation are less responsive to diet fat than lipogenesis (Lavau et al., 1979). However, as reported in Chapter IV (Figures IV-2 and IV-3), feeding a high fat (20% w/w) P/S=1.0 diet to diabetic animals, as compared to a high fat P/S=0.25 diet, significantly improved both adipocyte glucose oxidation and lipogenesis. However, the amount of glucose oxidized and incorporated into lipids by animals fed the high fat P/S=1.0 diet was similar to the amounts observed for diabetic animals fed the low fat high P/S diet in the present study (Figures VIII-2 and VIII-3). Alterations in rate of glucose transport cannot explain the improved lipogenic capacity of cells from diabetic animals fed the low fat high P/S diet observed in the present study. Differences in membrane fatty acid composition in the diabetic state may help explain the different response of cells from diabetic, compared to control animals; in Chapters III and IV, feeding a high P/S diet attenuated, but did not completely normalize, some of the predicted diabetes associated changes in the polyunsaturated and monounsaturated fatty acid composition of membrane phospholipids.

Although alterations in the membrane environment offer a possible explanation for the response to diet and diabetes observed, other factors cannot be overlooked. Caloric intake and cell size, although not measured, likely differ between treatment and diet groups and could influence cellular metabolism of glucose. In the present study, utilization of glucose was assumed to be a sum of the rates of lipogenesis and oxidation which neglect additional products produced in adipocytes such as lactate and glycogen. Although these pathways are quantitatively small in adipocytes, it was reported that when cells are incubated in high glucose concentrations (10 mM), high fat diets increase the incorporation of glucose into lactate and glycogen in adipocytes (Susini & Lavau, 1978). Moreover, the Dole extraction procedure used to extract lipids in the present study is specific for nonesterified long chain fatty acids (Dole, 1960). The single extraction Dole procedure used in the present study does not allow for accurate determination of insulin stimulated glucose incorporation into other products such as cholesterol and phospholipids. Recovery of these products by this procedure was estimated to be 4-44% (Dole, 1960). The synthesis of several of these products is quantitatively small (estimated to be approximately 10% of total incorporation) but synthesis of these products has been demonstrated to be stimulated by insulin and increased by feeding high saturated fat diets (Susini & Lavau, 1978).

The present study demonstrates that both the content and composition of dietary fat, when fed in proportions similar to segments of the North American population, alters the rates of insulin stimulated glucose transport and metabolism in epididymal adipocytes. The effect of diet fat on glucose metabolism depends to some extent on the pathway measured and may be modified by the diabetic state. Although the mechanisms by which dietary fat influences cellular insulin action are not clear, the effect of diet fat on the composition and structure of cell membranes offers a logical hypothesis to explain at least part of the known effects of diet-induced alterations in insulin responsiveness.

Table VIII.1 Fatty acid composition of diets fed.

Fatty Acid	High Fat P/S=0.2	Low Fat P/S=0.2 (% w/w of total fatty acids)	Low Fat P/S=2.0
$C_{14:0}$	2.8	3.0	.9
$C_{14:1}$	.1	.2	0
$C_{15:0}$	.5	.5	0
$C_{16:0}$	23.9	24.6	12.0
$C_{16:1}$	.4	.4	.2
$C_{17:0}$	1.8	1.9	.6
$C_{18:0}$	49.7	51.9	16.4
$C_{18:1}$	4.3	3.8	11.2
$C_{18:2(6)}$	13.9	10.1	55.1
$C_{18:3(6)}$	.2	.2	.1
$C_{18:3(3)}$	1.1	1.9	1.9
$C_{20:0}$	.1	.3	.4
$C_{20:1}$	0	0	.2
$C_{22:0}$	.1	.1	.1
Σpolyunsats	15.9	12.7	57.7
Σ(*w*-6)	14.3	10.6	55.5
Σ(*w*-3)	1.5	2.1	2.1
Σsats	78.8	82.2	30.4
Σmonounsats	4.8	4.4	11.7
P/S ratio	0.2	0.2	1.9

Fatty acid composition of diets as determined by gas liquid chromatography. Abbreviations used: Σpolyunsats, sum of polyunsaturated fatty acids; Σ(*w*-6), sum of (*w*-6) unsaturated fatty acids; Σ(*w*-3), sum of (*w*-3) unsaturated fatty acids; Σmonounsats, sum of monounsaturated fatty acids; Σsats, sum of saturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio.

Table VIII-2. Animal weights, serum glucose and insulin levels.

Diet Treatment	P/S	(n)	Body Weight (g)	Weight Gain (g/day)	Final Serum Levels of Glucose ((mg/dl)	Insulin (μU/ml)
Control Animals						
High fat	low	(11)	175± 7	5.2±.2[a]	141± 4[a]	46±12[a]
Low fat	low	(11)	192± 5	6.2±.2[b]	138± 4[a]	77± 9[a]
Low fat	high	(9)	206± 8	6.2±.1[b]	143± 7[a]	68± 6[a]
Diabetic Animals						
High fat	low	(8)	193± 7	1.7±.4[c]	507±34[b]	15± 2[b]
Low fat	low	(8)	194± 4	2.7±.4[d]	544±74[b]	16± 3[b]
Low fat	high	(8)	214±10	3.2±.5[d]	538±53[b]	24± 5[b]

Values are group means ± S.E. Body weight is weight 3 weeks after initiation of diet at the time of streptozotocin/placebo treatment. Treatments without a common superscript are significantly different ($p<0.05$). 1 ng insulin=25 μU.

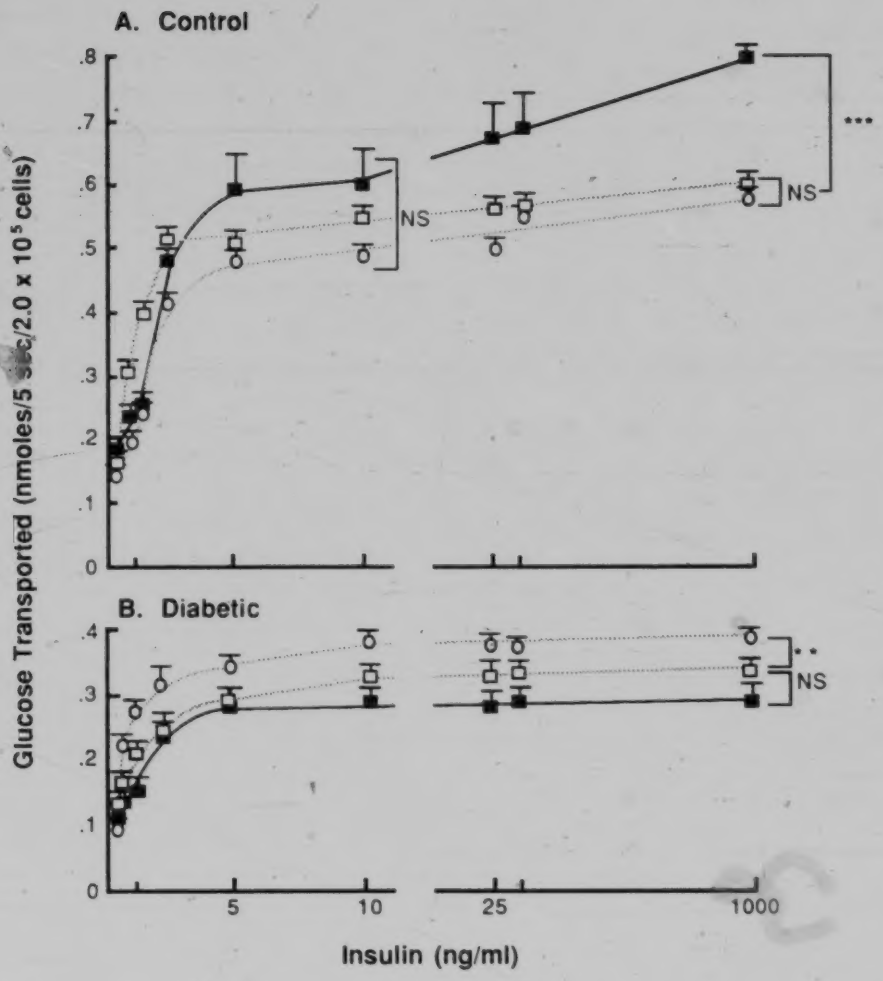

Figure VIII-1: Effect of dietary fat and diabetic state on insulin stimulated glucose transport.

Insulin stimulated glucose transport at .5mM glucose 36°C in adipocytes from (A) control animals and (B) diabetic animals fed: the high fat low P/S diet (o—o); the low fat low P/S diet (□·····□); and the low fat high P/S diet (■—■). Values are group means ± S.E (n/group as described in Table 1). The level of significance is indicated (**$p<0.01$; ***$p<0.001$). The maximum amount of glucose transported (1000 ng/ml insulin) under identical experimental conditions by cells from control and diabetic animals fed high fat (20% w/w) diets P/S=2.0 as illustrated in Chapter V, Figure V-3 was 1.02 ± .12 and .43 ± .03 nmoles/5 sec/20 x 10^5 cells, respectively. For both control and diabetic animals the amount of glucose transported by animals fed the high P/S diet was significantly greater than the amount transported by treatment-matched animals fed the low P/S diet (Chapter V).

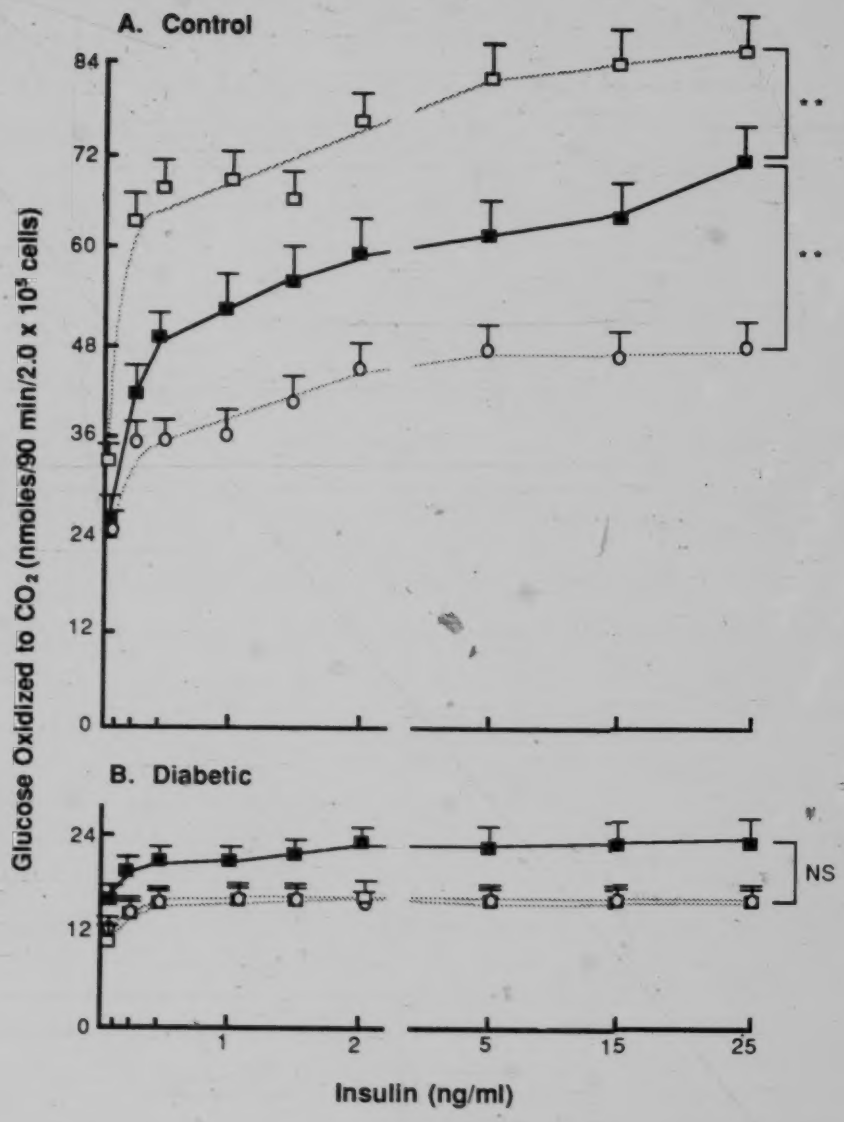

Figure VIII-2. Effect of dietary fat and diabetic state on insulin stimulated glucose oxidation.

Insulin stimulated glucose oxidation to CO_2 at 1.0mM glucose, 36°C in adipocytes from (A) control animals and (B) diabetic animals fed: the high fat low P/S diet (o—o); the low fat low P/S diet (□—□); and the low fat high P/S diet (■—■). Values are group means ± S.E. (n/group as described in Table 1). The level of significance is indicated (**$p<0.01$). The maximum amount of glucose oxidized (25ng/ml insulin) under similar experimental conditions by cells from control and diabetic animals fed a high fat P/S=1.0 diet, as illustrated in Chapter IV, Figure IV-2, was 32.7±4.8 and 21.8±2.4 nmoles/90 mins/2.0x10^5 cells, respectively. The amount of glucose oxidized by cells from diabetic animals fed the high fat P/S=1.0 diet was significantly greater than animals fed a high fat P/S=0.25 diet. Diet P/S did not affect the amount of glucose oxidized by control cells (Chapter IV).

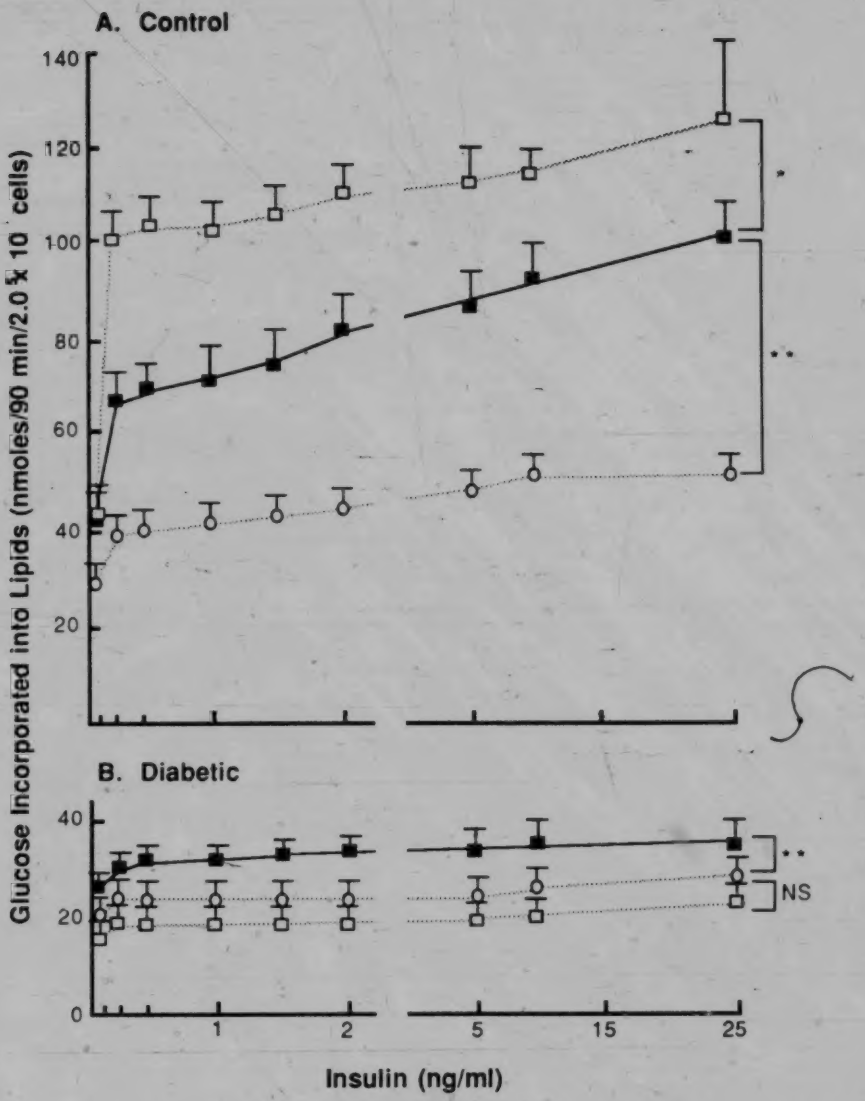

Figure VIII-3. Effect of dietary fat and diabetic state on insulin stimulated glucose incorporation into lipids.

Insulin stimulated glucose incoporation into lipids at 1.0mM glucose, 36°C in adipocytes from (A) control animals and (B) diabetic animals fed: the high fat low P/S diet (o—o); the low fat low P/S diet (□—□); and the low fat high P/S diet (■—■). Values are group means ± S.E (n/group as described in Table 1). The level of significance is indicated (*$p<0.05$; **$p<0.01$). The maximum amount of glucose incorporated into lipids (25ng/ml insulin) under similar experimental conditions by cells from control and diabetic animals fed a high fat P/S=1.0 diet, as illustrated in Chapter IV, Figure IV-3, was 56.7±1.7 and 45.0±3.8 nmoles/90 mins/2.0x10^5 cells, respectively. The amount of glucose incorporated into lipids was significantly greater for both diabetic and control animals fed the P/S=1.0 diet, compared to treatment-matched animals fed the high fat P/S=0.25 diet (Chapter IV).

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IX. THE RELATIONSHIP BETWEEN DIETARY FAT, ADIPOCYTE MEMBRANE COMPOSITION AND INSULIN BINDING [1]

A. INTRODUCTION

Current understanding of the organization of biological membranes relies on the fluid mosaic model proposed by Singer and Nicholson (1972) in which proteins are embedded to varying degrees in a lipid bilayer. The adipocyte insulin receptor is located on the cell surface, acting within the lipid matrix of the plasma membrane (Jarett & Smith, 1974). Because of the intimate contact of the receptor with membrane lipids it is conceivable that alterations in the composition or physical state of adjacent phospholipids influence cellular insulin action. The function of other membrane associated proteins (Hargreaves & Clandinin, 1987a and 1987b; Garg et al., 1988), including hormone-mediated function (Morson & Clandinin, 1985; Neelands & Clandinin, 1983; Clandinin et al., 1985), have been clearly demonstrated, in a variety of tissues, to be altered by changes in membrane lipid composition.

A number of studies have indirectly suggested that the physical state of the membrane is important for insulin binding (Cuatrecasas, 1971; Rodbell, 1966; Amatruda & Finch, 1979). Subjecting the plasma membrane to treatments that disrupt the protein and lipid components such as trypsin (Cuatrecasas, 1971), benzyl alcohol (Hyslop et al., 1987) and phospholipase A and C (Rodbell, 1966) reduce insulin binding and hormone mediated responses. Temperature-induced alterations in binding have been interpreted to result from changes in membrane fluidity (Amatruda & Finch, 1979). The concept of membrane fluidity refers more specifically to the properties of the hydrophobic core of the membrane. These properties depend not only on temperature but also the chemical composition of the membrane, mainly its cholesterol and phospholipid content and the length and degree of unsaturation of the fatty acyl chains of the phospholipid core. *In vitro*, cell culture (Ginsberg et al., 1981; Grundfeld et al., 1981) and reconstitution (Gould et al., 1982) studies suggest that membrane lipid composition influences the binding properties of the insulin receptor. In these studies a more

[1] A version of this chapter has been submitted for publication. Field CJ & Clandinin MT. (1988) Biochem J

polyunsaturated fatty acid composition was associated with increased membrane fluidity and an increased number of available insulin binding sites (Ginsberg et al., 1981; Grunfeld et al., 1981; Gould et al., 1982).

Although it has been established that composition of diet fat influences the composition of stored and structural lipids in adipose tissue (Field & Clandinin, 1984; Field et al., 1985), the effect of diet-induced alterations in membrane lipid on the function of this organ have, until recently, received little attention. There is some evidence that diet related alterations in membrane composition can influence insulin binding. Highly polyunsaturated membranes, demonstrating enhanced insulin binding, were produced by growing Ehrlich ascites cells in mice fed diets containing a high level of polyunsaturated fat, compared to monounsaturated fat (Ginsberg et al., 1982). Difficulties, however, exist in applying this study to man; the ratio of polyunsaturated to saturated fatty acids were clearly unphysiological with respect to diets habitually consumed by man (Field et al., 1985). In Chapters III, VI and VII it was demonstrated that feeding high fat diets (20% w/w), with high but physiological P/S ratios of 1.0 or 2.0, compared to low P/S ratios of 0.25 or 0.20, were associated with an increased polyunsaturated fatty acid content in the major adipocyte membrane phospholipid fractions and enhanced insulin binding (Chapters III and V). The following study was designed to test the hypothesis that a direct relationship exists between the P/S ratio of dietary fat consumed, the composition of the major adipocyte plasma membrane phospholipids and insulin binding. Determination of this relationship would enable identification of specific alterations in membrane composition that modify this membrane function.

B. METHODS

Animals and Diets

Twenty weanling male Sprague-Dawley rats (47.9±.4g) were randomly assigned to one of ten high fat (20% w/w) diet groups. The nutrient composition of the high fat diet is

reported in Chapter III. The ten diets differed in the proportions of safflower oil and hydrogenated beef tallow to provide polyunsaturated to saturated fatty acid (P/S) ratios from 0.14 to 1.8. Linseed oil was added to all diets to ensure an adequate intake of *w*-3 fatty acids (1% w/w of the diet fat). All diets were cholesterol free by analysis. The fatty acid composition of the ten diets as analyzed by gas liquid chromatography is presented (Table IX-1).

The diets were fed *ad libitum* to animals for 6 weeks. One animal per diet treatment was sacrificed on each of two days by decapitation and serum collected for glucose and insulin determinations as described in Chapter III. Epididymal fat pads were collected and adipocytes isolated as described in Chapter III.

Insulin Binding

Insulin binding to isolated adipocytes was measured at 5 insulin concentrations (0.4, 1.2, 10, 100, and 1000 ng/ml) and corrected to cell number as described in Chapter V.

Statistical Analysis

The relationship between diet P/S, membrane composition and insulin binding was examined by regression analysis (Steel & Torrie, 1980).

C. RESULTS

The mean final weight and weight gain/day for the 20 animals was 364±7 g and 7.5±.2 g, respectively. Serum glucose was 139±3 mg/dl and serum insulin 60±9 μU/ml. No significant correlations were found between diet and body weight or serum glucose or insulin values.

To examine the relationship between diet P/S, membrane composition and insulin binding, regression lines were constructed between diet P/S ratio and insulin binding for the five insulin concentrations measured (Figure IX-1) and between diet P/S and the major fatty acid content of membrane phospholipids (Figure IX-2). A significant ($p<0.05$) positive

relationship was found between the P/S ratio of the diet and the amount of insulin bound at each of the five insulin concentrations examined (Figure IX-1). The diet P/S ratio influenced the content (w/w) of the major membrane fatty acids in adipocyte membrane phospholipids as illustrated by the mean phospholipid fatty acid composition of the lowest (P/S=0.14) and highest (P/S=1.8) P/S ratio in the diets fed (Table IX-2). The content of several fatty acids in phosphatidylcholine and most of the major fatty acids in phosphatidylethanolamine were found to be significantly related to change in the diet P/S ratio and the amount of insulin bound at insulin concentrations of 0.4, 1.2, 10 and 100 ng/ml (Table IX-2). For example, diet P/S was found to be positively related to the *w*-6 fatty acid content of phosphatidylethanolamine and the $C_{18:0}$ content of phosphatidylcholine (Figure IX-1). A negative relationship was observed between diet P/S and the saturated and monounsaturated fatty acid content of phosphatidylethanolamine and the $C_{16:0}$ content of phosphatidylcholine (Figure IX-2).

To examine the relationship between membrane composition and insulin binding, regression lines were constructed between plasma membrane phospholipid fatty acid content and insulin binding at four insulin concentrations (Figure IX-3). The relationship between insulin binding and membrane lipid composition (Figure IX-3) for specific fatty acids was found to be in the same direction as that observed between membrane composition and diet P/S (Figure IX-2). The amount of insulin bound increased as the content of *w*-6 fatty acids in phosphatidylethanolamine and the $C_{16:0}$ in phosphatidylcholine increased. Insulin binding decreased with increasing content of saturated and monounsaturated fatty acids in phosphatidylethanolamine and $C_{18:0}$ in phosphatidylcholine (Figure IX-3). Although not illustrated, insulin binding and diet P/S were also found to be positively related to the $C_{18:2(6)}$, total polyunsaturated fatty acid content and P/S ratio of phosphatidylethanolamine and negatively related to $C_{16:1}$ levels in phosphatidylcholine, $C_{16:0}$ and $C_{18:1}$ levels in phosphatidylethanolamine, and to $C_{16:1}$ content of phosphatidylserine (Table IX-2).

D. DISCUSSION

Previous studies have demonstrated that hormone binding (Ginsberg et al., 1981; Gould et al., 1982; Ginsberg et al., 1982) and binding mediated functions (Sandra & Fyler, 1981; Neelands & Clandinin, 1983) are influenced by the fatty acyl composition of cell membranes. Increasing the unsaturation index of phospholipids by feeding diets containing different levels of unsaturated fatty acids was shown to be positively related to glucagon stimulated adenylate cyclase activity in the liver plasma membrane (Neelands & Clandinin, 1983). Feeding high P/S diet as compared to low P/S diet was associated with increased polyunsaturated fatty acyl composition of adipocyte membrane phospholipids (Chapters II and VI) and enhanced insulin binding (Chapter III and V).

In the present study feeding high fat diets (20% w/w), providing P/S ratios representative of the range observed in the human diet (Field et al., 1985), significantly altered the fatty acyl composition of membrane phospholipids in a linear dose dependent manner (Figure IX-2). Increasing the dietary P/S ratio was associated with increases in the polyunsaturated fatty acid content of phosphatidylethanolamine and decreases in some of the major monounsaturated fatty acids present in phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine (Figure IX-2; Table IX-2). These diet-induced effects on membrane phospholipids are consistent with those reported in Chapters VI and VII. In the present study specific diet-induced transitions in membrane phospholipid fatty acid composition were paralleled by changes in insulin binding at both physiological and supra-physiological insulin concentrations (Table IX-2; Figure IX-3).

The findings of the present study are consistent with previous reports *in vitro* where insulin binding was found to be altered by profound manipulations in membrane composition induced either by substituting culture media with specific lipids (Ginsberg et al., 1981) or reconstitution of the receptor in lipid vesicles (Gould et al., 1982). These studies reported increased insulin binding in a more polyunsaturated, compared to monounsaturated (Ginsberg et al., 1981) or saturated (Gould et al., 1982), membrane environment. It is, however, difficult to compare studies of *in vitro* fatty acid manipulation to the results of the present *in*

vivo study as *in vitro* usually only one or two fatty acids are manipulated and very large and likely unphysiological alterations in membrane composition are induced. Changing the nature of diet fat clearly manipulates membrane content of more than one fatty acid and induces specific alterations in membrane phospholipid fractions (Clandinin et al., 1985; Neelands & Clandinin, 1983; Table IX-1).

The molecular mechanisms by which membrane fatty acid alterations influence insulin binding are not clear. Gould et al. (1982) proposed that the accessibility of the insulin receptor for its ligand may be controlled by conformational changes in the receptor that require a more fluid environment and cannot take place when the phospholipid fatty acyl chains surrounding the receptor are saturated. Alternatively, it was suggested that the receptor may exist in two states; a monomeric state of high binding capacity, low ligand affinity and a polymeric state of low binding capacity, high affinity; and that the level of membrane saturation may determine which state is favoured (Ginsberg et al., 1982).

Reconstitution studies suggest that insulin binding relies to some extent on the phospholipid content of the membrane (Sandra & Fyler, 1981). In Chapter VII it was found that alterations in the diet P/S ratio were also associated with changes in the content of several phospholipids including phosphatidylethanolamine (Table VII-7). This may be of particular significance, since, in the present study, the content of most of the major fatty acids in phosphatidylethanolamine were found to directly relate to insulin binding. Although the exact mechanism by which diet fat alters membrane composition is unknown, diet fat modifications have been demonstrated to alter the *de novo* synthesis of phospholipids (Hargreaves & Clandinin, 1987a), redistribution of fatty acyl chains via phospholipase (Van den Bosch, 1980) or acyl transferases (Hargreaves & Clandinin, 1987b), and desaturation of membrane phospholipid fatty acids (Garg et al., 1988).

The diabetic state is associated with specific alterations in membrane lipid composition (Chapters III, VI, and VII; Faas & Carter, 1980) that can be significantly modified by alterations in diet fat (Chapters III, VI and VII). Based on the findings of the present study, the increased polyunsaturated and decreased monounsaturated fatty acid composition

associated with the diabetic state (Faas & Carter, 1980; Chapter VI, VII) would be consistent with improved insulin binding. The existence of an alteration in adipocyte insulin binding despite clear resistance to insulin action in human diabetes is, at present, still controversial (Sinha et al., 1987; Arner, 1987). The streptozotocin diabetic rat has been reported to exhibit increased insulin binding compared to control animals (Schoenle et al., 1977). However, in Chapters III and V no apparant effect of diet on insulin binding was found in cells from diabetic animals, despite clear alterations in insulin stimulated action. This observation suggests that; 1. diet fat-induced effects on insulin action in this diabetic model may not occur at the level of insulin binding; 2. the degree of change in membrane composition that occurs in the diabetic state is too great to be counteracted by the modest change in diet fat composition tested.

In control animals, feeding high fat high polyunsaturated fat diets, as compared to saturated fat diets, improved cellular insulin action (Awad, 1981; Chapters IV and V). The present study suggests that increasing the polyunsaturated fatty acid content of the diet increases adipocyte insulin binding by changing the surrounding lipid environment, thereby demonstrating a clear physiological mechanism by which a high P/S diet may improve insulin stimulated action in adipose tissue. The importance of this mechanism in other insulin sensitive tissues and physiological states, such as obesity, remains to be assessed.

Table IX-1. Major fatty acid composition of diets.

P/S =	Diets (% w/w composition) 0.14	0.35	0.54	0.74	0.92	1.10	1.33	1.52	1.66	1.81
FATTY ACID										
$C_{14:0}$	3.5	2.9	2.4	2.1	1.9	1.7	1.5	1.3	1.2	1.2
$C_{16:0}$	26.2	22.6	20.2	18.2	16.8	15.9	14.7	13.8	13.4	13.0
$C_{16:1}$	.4	1.0	.8	.7	.6	.5	.5	.4	.4	.2
$C_{18:0}$	50.1	40.7	35.1	30.1	26.7	23.6	20.9	19.1	17.4	16.3
$C_{18:1}$	3.6	5.3	6.7	7.5	8.2	8.8	9.4	9.8	10.1	10.7
$C_{18:2(6)}$	10.7	22.9	31.5	37.6	42.2	46.2	49.9	52.9	54.5	55.9
$C_{18:3(3)}$	.8	1.0	1.1	1.0	1.0	0.9	1.0	1.0	1.0	1.0
Σpolyunsats	11.8	24.1	32.6	38.8	43.3	47.3	51.1	54.0	55.7	57.1
Σw-6	10.9	23.1	31.5	37.7	42.3	46.3	50.0	53.0	54.6	56.0
Σw-3	0.9	1.0	1.1	1.0	1.0	0.9	1.1	1.0	1.1	1.1
Σsats	83.0	69.0	60.0	52.3	47.1	42.8	38.5	35.5	33.3	31.6
Σmonounsats	5.2	6.6	7.4	8.6	9.3	9.6	10.2	10.3	10.9	11.3

Major fatty acid composition of diets as determined by gas liquid chromotography. Abbreviations used Σpolyunsats, sum polyunsaturated fatty acids; Σw-6, sum (w-6) unsaturated fatty acids; Σw-3,sum of (w-3) unsaturated fatty acids; Σsats, sum of saturated fatty acids; Σmonounsats, sum of monounsaturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio.

Table IX-2. Effect of diet P/S on the fatty acyl composition of adipocyte plasma membrane phospholipids.

	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol		Phosphatidylserine	
	(% w/w)							
	Diet P/S							
Fatty Acid	0.14	1.8	0.14	1.8	0.14	1.8	0.14	1.8
$C_{16:0}$	22.5	15.7*+	24.7	13.3*+	15.4	12.9	20.5	7.5
$C_{16:1}$	2.9	0.4*+	2.7	1.6	2.7	2.2	3.2	0.6*+
$C_{18:0}$	27.8	34.6*+	19.4	23.6*+	30.4	33.1	26.2	35.1
$C_{18:1}$	8.3	6.1	19.0	13.8*+	8.4	9.4	9.1	7.3
$C_{18:2}$	20.0	28.7	8.5	17.4*+	5.9	9.9	10.2	21.3
$C_{20:4(6)}$	10.0	11.4	10.7	19.1	16.5	23.0	7.8	15.4
sats	52.0	51.8	49.5	39.0*+	52.6	48.5	51.4	46.4
monounsats	11.8	6.8	25.2	19.8*+	16.0	15.2	15.5	10.7
polyunsats	36.2	41.2	25.2	40.7*+	31.1	36.3	33.2	45.0
Σ(ω-6)	34.5	40.8	22.3	39.2*+	27.9	34.8	30.9	42.3
Σ(ω-3)	1.7	0.9	2.7	1.5	3.2	1.5	2.3	2.7
P/S	.67	.80	.50	1.04*+	.59	.75	.65	.97

Values are the mean (n=2/group) composition of the major fatty acids of the adipocyte membrane for animals fed the lowest (P/S=0.14) and highest (P/S=1.8) of the ten experimental diets, differing in P/S ratios. Abbreviations as defined in Table IX-1. Significant linear regression of the ten treatment ($p<0.05$) is indicated; (*) diet P/S and phospholipid fatty acid composition; and (+) membrane fatty acid composition and insulin bound at four insulin concentrations (.4, 1.2, 10 and 100 ng/ml).

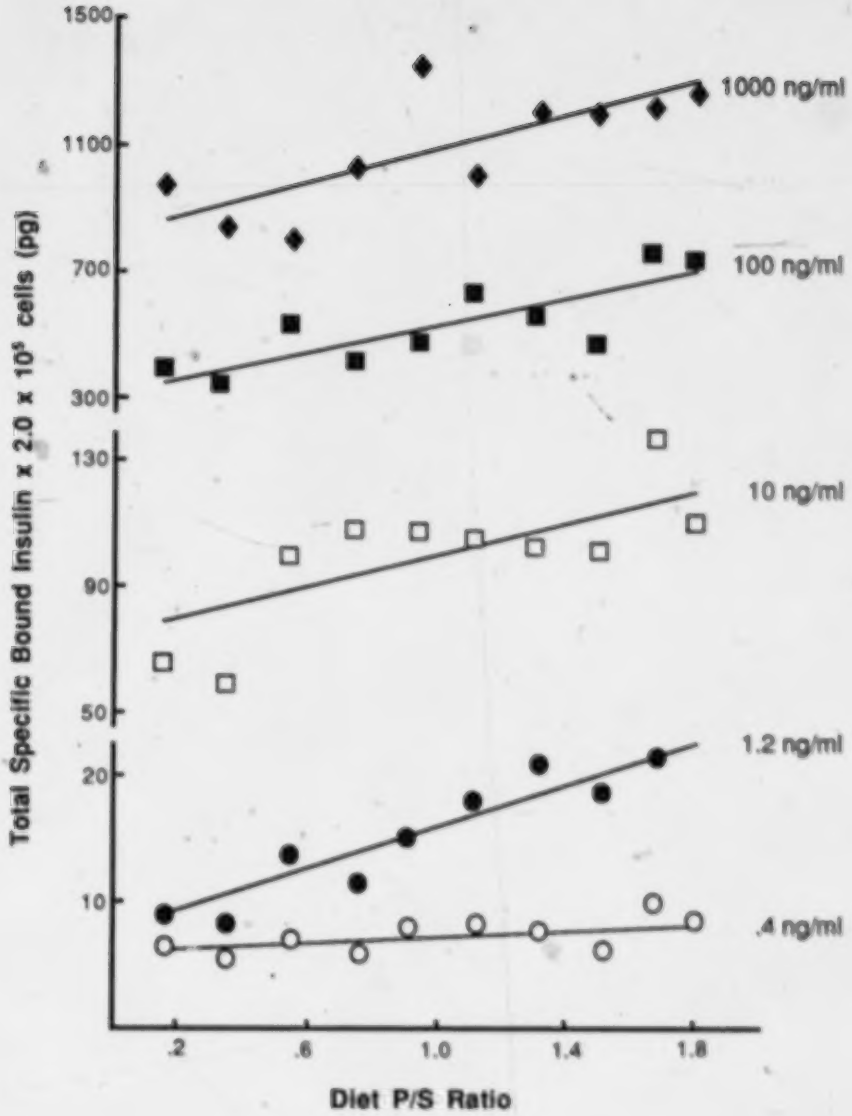

Figure IX-1. Relationship between diet P/S and insulin binding.

Regression lines were constructed for dietary P/S ratio verses the mean (n=2/group) amount of insulin bound at the five insulin concentrations measured (4, 1.2, 10, 100 and 1000 ng/ml). All regression coefficients were significant ($p<0.05$).

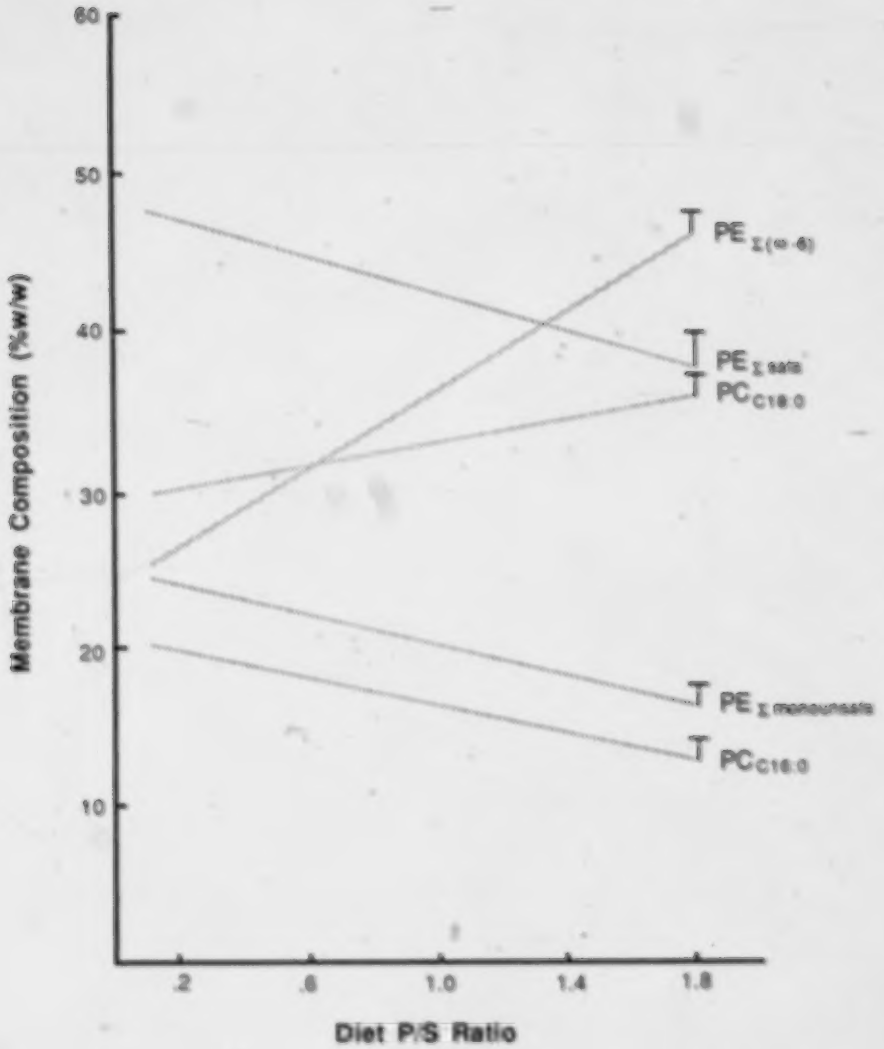

Figure IX-2. Relationship between diet P/S and and membrane composition.

Regression lines were constructed for diet P/S verses the mean (n=2/group) fatty acid content (% w/w) of adipocyte membrane phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by animals fed the ten different P/S diets. All regression coefficients were significant ($p<0.05$). Abbreviations as defined in Table IX-1.

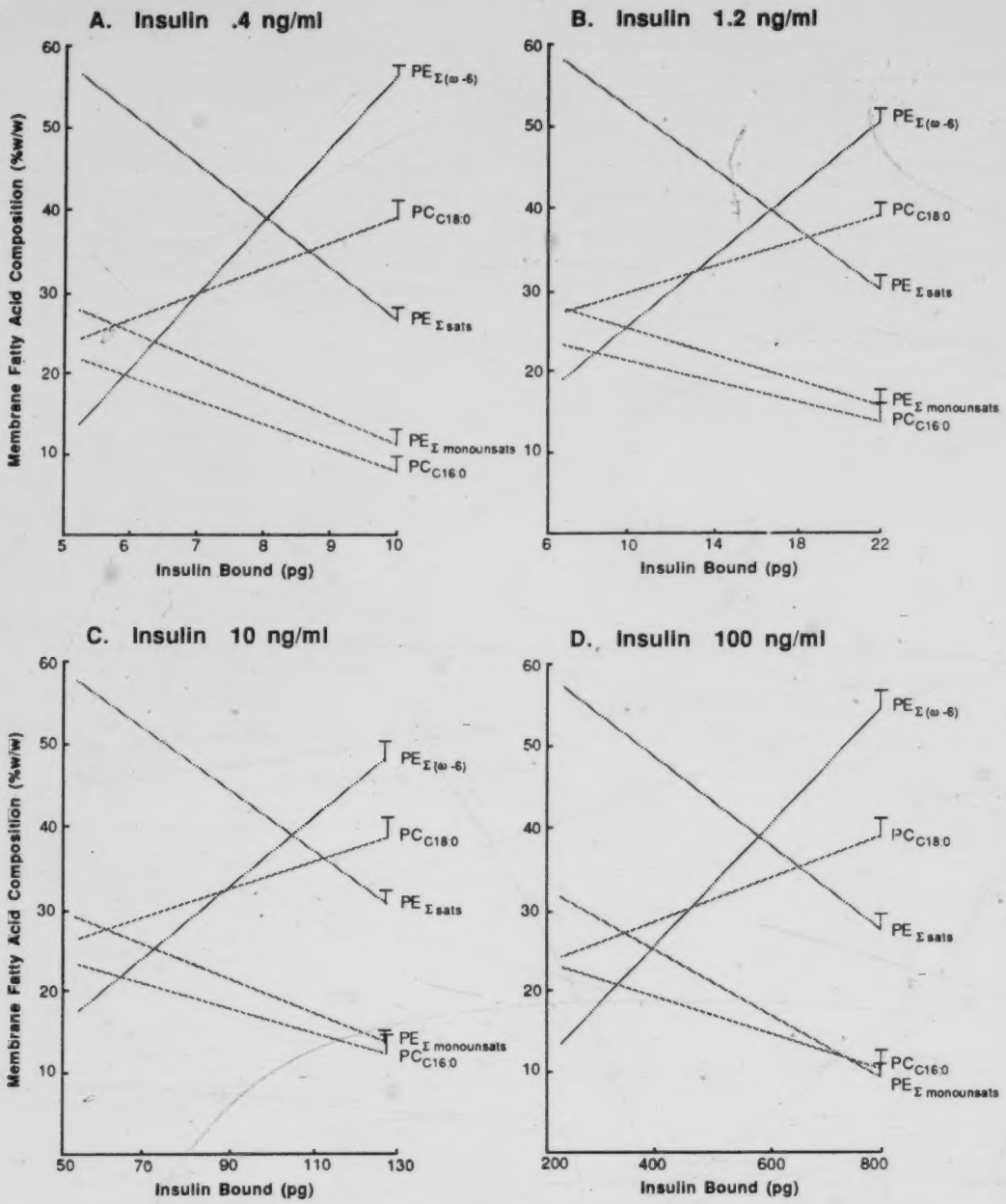

Figure IX-3. Relationship between insulin binding and the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine.

Regression lines were constructed for fatty acid content % (w/w) of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) versus the amount of insulin bound at insulin concentrations; A, .4 ng/ml; B, 1.2 ng/ml; C, 10 ng/ml; D, 100 ng/ml. All regression coefficients were significant ($p<0.05$). Abbreviations as defined in Table IX-1.

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X. THE EFFECT OF DIETARY FAT CONTENT AND COMPOSITION ON ADIPOCYTE LIPIDS IN NORMAL AND DIABETIC STATES [1]

A. INTRODUCTION

There is convincing evidence in both man (Field et al., 1985; Wallidus et al., 1980) and animals (Field & Clandinin, 1984; Ostwald et al., 1962; Eisen et al., 1982; Beare & Kates, 1964; Herodek & Csakvary, 1972; Pavey et al., 1976) that the nature of fat consumed influences the fatty acid composition of body fat. As mammals depend on an exogenous supply of essential fatty acids, the presence of these fatty acids in adipose tissue can be assumed to originate from the diet. For this reason, research on adipose tissue composition has focused on the effect of fat intake on the level of linoleic acid in body fat depots. Moreover, mammals have ability to synthesize fatty acids from non-lipid precursors and to modify ingested fatty acids by desaturation, thus dietary fat intake is not the sole factor determining the composition of non-essential fatty acids in stored lipids. The relationship between diet fat and adipose tissue composition appears to be complicated by a variety of physiological and dietary factors (reviewed by Field & Clandinin, 1984).

The role of dietary fat level on *de novo* fat synthesis and storage is not clear. There is general concensus in liver that high fat diets, particularly those high in polyunsaturated fat, suppress *de novo* fatty acid synthesis (Clark et al.,1977). Early work by Romsos and Leveille (1974) in meal-fed rats suggests that as much as 70% of the *de novo* fatty acid synthesis in this animal takes place in the adipose organ. Recently it was demonstrated that for rats fed high fat diets, adipose tissue can continue to be a source of *de novo* fatty acid synthesis (Nelson et al., 1987). Moreover, substituting saturated for polyunsaturated fats did not significantly alter the rate of fatty acid synthesis (Nelson et al., 1987).

In the diabetic condition alterations in tissue fatty acid composition have been reported in both animals (Worcester et al., 1979; Faas & Carter, 1980; Benjamin & Gellhorn, 1964; Friedman et al., 1966) and humans (Van Doormal et al., 1984), suggesting abnormal

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fatty acid metabolism. These alterations have been related to diminished rates of Δ6 and Δ9 desaturation in diabetic animals (Faas & Carter, 1980; Worcester et al., 1979; Benjamin & Gellhorn, 1964). Insulin therapy apparently corrects the defects in desaturase activity but only partially normalizes the altered fatty acid composition (Faas & Carter, 1980; Van Doormal et al., 1984). Feeding high fat diets, especially those high in polyunsaturated fats inhibit Δ9 and Δ6 desaturase enzymes (Wahle & Radcliffe, 1977; Weekes et al., 1986). The potential role of diet in attenuating diabetic-induced changes in desaturase activity has not been identified, possibly due to the use of experimental diets differing in the level and type of fatty acids or the use of essential fatty acid deficient diets in previous studies. The physiological implications of a change in desaturase activity and level of unsaturated fatty acids in membrane phospholipids are not clear, however, it has been suggested that alterations in membrane lipid composition may be involved in the etiology of the insulin resistance (Sandra & Fyler, 1982).

Current dietary guidelines for both the general population and the nutritional management of diabetes recommend that total fat intake be reduced to 30% of energy and an effort made to replace saturated fats with polyunsaturated fats (American Diabetes Association, 1987; Kissebah & Schectman, 1988). The physiological consequences of these dietary changes are not well understood. In Chapter VII, it was demonstrated that both the content and composition of diet fat altered the content and composition of adipocyte membrane phospholipids. Quanitatively, the adipose organ represents a significant supply of fatty acids for the *de novo* synthesis of compounds such as phospholipids. Thus, the following study was conducted to; 1. identify the effect of both the content and composition of dietary fat on the composition of adipocyte stored lipids; 2. define the role of diet fat in amplifying or attenuating the expected changes in fatty acid composition associated with the diabetic state.

B. METHODS

Animals and Diets

Sixty weanling male Sprague-Dawley rats (59.7±2.1g) were randomly assigned to one of four diet groups. Two low fat (10% w/w) and two high fat (20% w/w) diets were fed. The nutrient composition of high and low fat diets are reported in Chapters VII and III and the fatty acid composition of the diets, as determined by gas liquid chromatography, illustrated in Chapter VII, Table VII-1. The experimental protocol was identical to that described in Chapter VII. Animals were sacrificed over a five day period by decapitation and serum was collected for glucose and insulin determinations. Epididymal fat pads were collected and adipocytes isolated as described in Chapter III.

Lipid Extraction and Analysis

Isolated adipocytes were resuspended in an equal volume of buffer (0.25M sucrose, 1mM EDTA in 10mM Tris-HCl, pH 7.4) at 4°C and homogenized in a 5ml ground glass homogenizer. The homogenate was centrifuged at 1054 g for 30 mins. A sample (approx. 0.25g) of the fat cake on top, containing the neutral lipids, was collected and extracted in 8ml chloroform:methanol (2:1 v/v) as described by Folch et al. (1957). The layers were split by the addition of 2 ml KCl (0.15M). The bottom layer containing neutral lipids was collected, dried under vacuum, then saponified and methylated as described by Bannon et al. (1982) using KOH and boron trifluoride methanol (14% w/v) reagents. Fatty acid methyl esters were separated and quantified by gas-liquid chromatography as described in Chapter III.

Statistical Analysis

The effect of diet fat content, diet P/S ratio and disease state on the major fatty acids in adipocyte stored lipids were compared by leasted-squared three way analysis of variance procedure (Harvey, 1975). Duncan's multiple range test was used to discriminate significant differences ($p<0.05$) in animal weight, serum glucose and serum insulin levels between

groups (Steel & Torrie, 1980).

C. RESULTS AND DISCUSSION

Animal Weight, Serum Glucose and Insulin

Diabetic animals gained significantly less weight and exhibited significantly higher serum glucose levels and lower serum insulin levels as illustrated in Chapter VII, Table VII-2. Animals fed the high fat low P/S diet during the initial three weeks of the experiment weighed significantly less than all other diet groups at the time of streptozotocin/placebo treatment. However, diet treatment did not affect weight, serum glucose or serum insulin levels in either control or diabetic groups after streptozotocin/placebo treatment.

Effect of Dietary Fat Content on the Composition of Adipocyte Neutral Lipids

The present study suggests that the quantity of fatty acid consumed influences the fatty acid composition of the adipocyte (Table X-1). High fat diets compared to low fat diets were associated with an increased content of polyunsaturated fatty acids (total polyunsaturated, total *w*-6 and $C_{18:2(6)}$ in adipocytes. The effect of fat level was particularly apparent in animals fed the P/S=0.2 diet that provided less $C_{18:2(6)}$ (P/S=0.2). On a body weight basis $C_{18:2(6)}$ intake was previously reported to relate to the essential fatty acid content of adipose lipids in both man (Field et al., 1985) and animals (Herodek & Csavary, 1972). In Chapter VII, in these animals a similar effect of diet P/S ratio on the polyunsaturated fat content of adipocyte membrane phospholipids was observed.

The monounsaturated fatty acid content ($C_{14:1}$ and $C_{16:1}$) was higher in lipid from both control and diabetic animals fed low fat diets. The increased monounsaturated fatty acid content (total monounsaturated, $C_{14:1}$, $C_{16:1}$ and $C_{18:1}$) associated with low fat diets was most dramatic in animals fed low fat low P/S diets. In tissues, $\Delta 9$ desaturase converts saturated fatty acids to monounsaturated fatty acids (Brenner, 1974). Although not as well studied as the hepatic enzyme, active $\Delta 9$ desaturation has been demonstrated in adipose tissue

(Wahle & Radcliffe, 1977). The hepatic $\Delta 9$ desaturase enzyme has been clearly shown to be inhibited by high fat diets, particularly those high in polyunsaturated fatty acids (Herodek & Csakvary, 1972; Weekes et al., 1986). A higher rate of desaturation in animals fed low fat low P/S diets may be responsible for the lower levels of saturated fatty acids (total saturated, $C_{14:0}$, $C_{15:0}$, $C_{17:0}$, and $C_{18:0}$) observed in adipose lipids. A higher monounsaturated fatty acid content may also be the result of an increased rate of endogenous fatty acid synthesis; changes in desaturase activity have been found to be parallelled by similar changes in fatty acid synthesis (Worcester et al., 1979). A higher content of $C_{16:0}$, a major end product of endogenous fatty acid synthesis, was observed in the present study in animals fed low as compared to high fat diets. A similar effect of fat level on the monounsaturated fatty acid composition of adipocyte membrane phospholipids was observed in these animals (Chapter VII).

Effect of Dietary P/S Ratio on the Composition of Adipocyte Neutral Lipids

Feeding high P/S diets, as compared to low P/S diets, significantly increased the polyunsaturated fatty acyl composition (total polyunsaturated fatty acids, total *w*-6 and $C_{18:2(6)}$) of adipocyte lipids in both control and diabetic animals (Table X-1). This observation is consistent with reports in man (Field et al., 1985; Wallidus et al., 1980) and animals (Ostwald et al., 1982; Eisen et al., 1982; Beare & Kates, 1964; Herodek & Csakvary, 1972; Pavey et al., 1976), suggesting that the polyunsaturated fatty acid composition of adipose tissue is directly related to the polyunsaturated fatty acid composition of diet fat. Based on some of the available human literature, a mathematical relationship has been established to predict the $C_{18:2(6)}$ content of adipose tissue from the estimated percent of this fatty acid in the diet (Beynen et al., 1980). There appears, however, to be a limit to the extent to which dietary $C_{18:2(6)}$ influences adipose tissue lipids as it was found that after $C_{18:2(6)}$ content in adipose tissue reached 60% (w/w) no further increase could be induced by diet (Beare & Kates, 1964).

A lower content of monounsaturated fatty acids (total monounsaturated, $C_{14:1}$, $C_{16:1}$, and $C_{18:1}$) and saturated fatty acids (total saturated fatty acids, $C_{14:0}$, $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$) was observed in animals fed high P/S, as compared to low P/S diets. This observation is consistent with previous reports of a progressive decrease in monounsaturated and saturated fatty acids as the polyunsaturated fatty acid content of the diet increases (Tove & Smith, 1960; Herodek & Csakvary, 1972). The high content of monounsaturated fatty acids in lipids from animals fed low P/S diets, low in monounsaturated fatty acids (Table VIII-2), suggests that unlike dietary $C_{18:2(6)}$, saturated fatty acids undergo considerable desaturation prior to storage.

As reported earlier (Field et al., 1985; Pavey et al., 1976; Ostwald et al., 1962; Wallidus et al., 1980) on a percent w/w basis very little $C_{20:4(6)}$ is stored in adipose tissue. However, considering the magnitude of the adipose organ, adipose tissue represents a significant storage depot for $C_{20:4(6)}$. Feeding a high P/S diet compared to a low P/S diet produced a greater than 3.5-fold increase in the $C_{20:4(6)}$ content in lipids from control animals and a greater than 2-fold increase in diabetic animals.

Effect of the Diabetic State on the Composition of Adipocyte Neutral Lipids

In the present study the diabetic state was associated with higher levels of several saturated fatty acids ($C_{15:0}$, $C_{17:0}$ and $C_{18:0}$). Lipids from diabetic animals fed high P/S diets, compared to diet-matched control animals, were lower in polyunsaturated fatty acids (total polyunsaturated, total *w*-6 and $C_{18:2(6)}$. In these animals the polyunsaturated fatty acid content was reduced to approximately 95% of the level in control animals. On a whole body basis this reduction in polyunsaturated fatty acids is magnified as body fat content is likely reduced in diabetic animals (Table X-2). These observations are consistent with previous reports in serum, plasma erythrocytes, liver and liver microsomes (Van Doormal et al., 1984; Faas & Carter, 1980; Worcester et al., 1979). The reduced content of monounsaturated fatty acids reported by others to accompany the diabetic condition (Van Doormal et al., 1984; Faas & Carter, 1980; Worcester et al., 1979) as a result of decreased

rates of Δ9 desaturation (Faas & Carter, 1980; Worcester et al., 1979), were not observed in the present study. However, a significant reduction in monounsaturated fatty acids in adipocyte membrane phospholipids were found in these animals (Chapter VII), suggesting that the defect in desaturation may be more visible in lipids that turnover more rapidly and undergo considerable fatty acid desaturation during synthesis.

The lower content of $C_{20:4(6)}$ in lipids from diabetic, as compared to control animals fed high P/S diets is consistent with previous reports of diminished rates of Δ6 desaturation in the diabetic state (Faas & Carter, 1980; Benjamin & Gellhorn, 1964). Although significantly lower than diet-matched control animals, the levels of $C_{20:4(6)}$ were still more than 2-fold greater in lipids from diabetic animals fed the high P/S, compared to low P/S, diets. If considered on a body basis, the amount of $C_{20:4(6)}$ stored in adipose tissue is considerably greater in diabetic animals fed the high P/S, as compared to low P/S diets (Table X-2). Feeding a high P/S diet to diabetic animals may, by increasing the amount of $C_{20:4(6)}$ available for membrane lipid synthesis, provide an effect buffer against the reported $C_{20:4(6)}$ deficiency in diabetic animals (Faas & Carter, 1980). In chapters III, VI and VII consistently higher levels of $C_{20:4(6)}$ were observed in membrane phospholipids from diabetic animals fed high P/S as compared to low P/S diets (Chapter III, VI, VII).

Physiological Implications of Altered Lipid Composition

In the present study the content and composition of dietary fat significantly altered the fatty acyl composition of epididymal adipocytes. As the adipose organ represents a major storage depot for fatty acids, dietary fat influences the total body pools of fatty acids available for synthesis of other complex membrane lipids. For example total adipose stores of essential fatty acids can be estimated (Table X-2). This estimation is based on the assumptions that approximately 10% of body weight in these rats is fat (Pullar & Webster, 1977) and the essential fatty acid composition of epididymal fat is representative of all body fat stores (reviewed by Field & Clandinin, 1984). From the values in Table X-2, it is evident that the content of $C_{20:4(6)}$ is lower in diabetic animals compared to control animals,

particularily animals fed high P/S diets. Although reduced by the diabetic state, the content of $C_{20:4(6)}$ in adipose tissue was higher and estimated total body stores of this essential fatty acid greater in diabetic animals fed the high P/S diets (Table X-2). Changes in the availablilty of fatty acids for the synthesis of membrane lipids may be partially responsible for the effect of dietary fat on the composition of adipocyte membrane phospholipids reported from these animals (Chapter VII).

The total *w*-3 fatty acid content, although relatively low in the diets fed (Table VII-1), was significantly altered by diet fat and the diabetic state. Linolenic acid $C_{18:3(3)}$ represented the major *w*-3 fatty acid in adipose tissue (Table X-2). Although $C_{20:5(3)}$ and $C_{22:6(3)}$ were detected in adipose tissue they represented less than 0.1% of adipose tissue fatty acids (data not shown). The total *w*-3 fatty acid content of adipose lipids was reduced by feeding a high fat high P/S diet and was approximately 10% lower in lipids from diabetic animals compared to diet-matched controls (Table X-1). As adipose tissue potentially represents a major supply of these fatty acids for membrane synthesis, the greater than 1.7-fold increase in total *w*-3 fatty acids in animals fed high fat low P/S diets is predicted to alter body stores of these essential fatty acids (Table X-2). These differences in *w*-3 fatty acids may be of physiological significance, as *w*-3 fatty acids have been postulated to have a role in insulin secretion and insulin sensitivity (Lardinois et al., 1987). In this regard, it was recently demonstrated that replacing 6% of the *w*-6 fatty acids in the diet with *w*-3 fatty acids prevented the insulin resistance associated with feeding high fat diets (Storlien et al., 1987).

The present study demonstrates that both the content and composition of dietary fat influence the composition of essential and non-essential fatty acids stored in the adipocyte. A higher polyunsaturated fatty acid composition was found in animals fed high fat and high polyunsaturated fat diets. A higher monounsaturated fatty acid composition was found in lipids from animals fed low fat diets and diets with low dietary P/S ratios. Compared to diet-matched control animals, the diabetic state was associated with a reduced content of $C_{20:4(6)}$, particularly in animals fed high P/S diets. The reduced body content of polyunsaturated fatty acids induced by the diabetic state and feeding low P/S diets may be of

physiological significance in terms of the availability of *w*-6 and *w*-3 fatty acids for the synthesis of membrane lipids.

Table X-3. Effect of diet fat and the diabetic state on the fatty acyl composition of adipocyte stored lipids.

	Composition (% w/w)								Significant Effect				
	High Fat				Low Fat								
	P/S=2.0		P/S=0.2		P/S=2.0		P/S=0.2						
Fatty Acid	C	D	C	D	C	D	C	D	F	P/S	D	FxP/S	DxP/S
$C_{14:0}$	1.6 ± .1	1.5 ± .1	4.6 ± .1	4.1 ± .1	1.8 ± .1	1.8 ± .1	3.6 ± .1	3.1 ± .4	•••	•••	•••	•••	•
$C_{14:1}$	.08±.01	.09±.01	.26±.01	.20±.01	.15±.02	.12±.01	.30±.02	.26±.02	•••	•••	•		
$C_{15:0}$	.23±.01	.22±.01	.59±.02	.60±.03	.24±.01	.24±.01	.42±.03	.36±.05	•••	•••	•••		
$C_{16:0}$	15.0 ± .5	17.0 ± .4	24.4 ± .7	24.7 ± .5	17.8 ±1.2	18.0 ± .8	27.0 ±1.0	26.5 ± .9	•	•••			
$C_{16:1}$	2.5 ± .2	2.4 ± .2	5.6 ± .3	4.0 ± .3	3.9 ± .5	3.6 ± .3	8.9 ± .5	8.5 ± .5	•••	•••	•	•••	
$C_{17:0}$	.33±.02	.40±.01	.81±.04	.93±.04	.33±.04	.36±.03	.50±.04	.54±.03	•••	•••	••	•••	
$C_{18:0}$	6.1 ± .6	7.1 ± .2	10.7 ± .8	13.5 ± .7	5.6 ± .6	6.3 ± .6	7.1 ± .5	7.5 ± .5	•••	•••	••	•••	
$C_{18:1}$	17.5 ± .3	17.5 ± .4	27.9 ± .7	26.2 ± .7	20.0 ± .4	21.2 ± .4	34.4 ± .4	32.7 ±1.7	•••	•••		••	
$C_{18:2(6)}$	54.2 ± .7	51.5 ± .7	22.0 ± .6	23.7 ± .9	47.0 ±1.7	45.5 ±1.3	15.4 ±1.0	16.4 ±1.0	•••	•••			•
$C_{18:3(3)}$	.84±.01	.73±.02	1.48±.04	1.35±.03	1.27±.06	1.20± .1	1.51±.07	1.37±.05	•••	•••	••	•••	
$C_{20:1(9)}$	.13±.02	.13±.01	.25±.02	.24±.04	.15±.02	.16±.02	.13±.01	.15±.03	•	••		•••	
$C_{20:4(6)}$	1.10±.10	.71±.03	.32±.02	.32±.03	.91±.06	.77±.05	.25±.02	.25±.03		•••	••		•
Σsats	23.1 ±1.0	26.3 ± .3	42.6 ± .7	43.9 ±1.0	25.6 ±1.5	26.5 ±1.0	38.7 ±1.1	38.2 ±1.1	•	•••		•••	
Σmonounsats	20.2 ± .5	20.3 ± .4	33.4 ± .5	30.5 ±1.0	24.7 ±1.0	25.3 ± .6	43.9 ± .8	43.3 ± .5	•••	•••		•••	
Σpolyunsats	56.5 ± .8	53.6 ± .7	23.7 ± .5	25.7± .6	48.2 ±1.9	48.3 ±1.2	17.5 ±1.0	18.5 ±1.1	•••	•••			•
Σ(w-6)	55.6 ± .8	52.7 ± .7	22.0 ± .4	24.2 ± .9	49.6 ±1.9	47.0 ±1.2	15.7 ±1.0	17.0 ±1.1	•••	•••			•
Σ(w-3)	.93±.01	.86±.01	1.64±.04	1.47±.04	1.42±.10	1.31±.08	1.67±.06	1.52±.05	••	•		••	
P/S	2.50±.15	2.04±.29	.56±.02	.59±.03	2.0 ±.20	1.85±.11	.46±.04	.49±.04		•••			•

Values are means ± S.E. (n/group as indicated in Table X-2). Abbreviations as defined in Table X-1. Significant effects by analysis of variance procedures of F, level of fat; P/S, dietary P/S ratio; D, diabetic state; and significant interactions are indicated; *p<0.05; **p<0.01; ***p<0.001. No threeway interactions were found.

Table X.2 Estimated total body stores of essential fatty acids.

Diet Treatment	P/S	Estimated total body content (g) $C_{18:2(6)}$	$C_{20:4(6)}$	$\Sigma(w\text{-}3)$
CONTROL ANIMALS				
High Fat	low	6.8	.10	.50
High Fat	high	19.5	.40	.33
Low Fat	low	5.3	.09	.57
Low Fat	high	17.5	.34	.53
DIABETIC ANIMALS				
High Fat	low	5.9	.08	.31
High Fat	high	15.1	.21	.25
Low Fat	low	4.9	.08	.45
Low Fat	high	12.7	.21	.37

An estimate of total body stores of each fatty acid was calculated by multiplying the amount of fatty acid found in adipose tissue (Table X-1) by an estimate of total body fat content. Total body fat content was assumed as 10% of body weight based on previously reported calculations for lean rats (Pullar & Webster, 1977). Inherent in these calculations are the assumptions that the percent body fat for diabetic rats is the same as normal and that all body fat depots are of similar essential fatty acid composition as epididymal fat pads.

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XI. SUMMARY AND DISCUSSION

The hypotheses tested in this thesis can be summarized as follows:

In both the normal and diabetic states, differences in the content and compositon of dietary fat will be reflected in:

1. the stored triglyceride content of the adipocyte;
2. the phospholipid content of the adipocyte plasma membrane; and
3. the fatty acyl composition of the major phospholipids of the adipocyte plasma membrane.

In both the normal and diabetic states, the composition of dietary fat will alter:

4. insulin binding;
5. glucose transport; and
6. intracellular glucose metabolism.

It was further hypothesized that:

7. diet-induced alterations in the fatty acyl composition of the adipocyte plasma membrane will relate to the integral membrane function of insulin binding.

These hypotheses have been verified as follows:

Hypothesis 1

Both the content and composition of dietary fat was demonstrated to influence the composition of essential and non-essential fatty acids stored in adipocytes from control and diabetic animals (Chapter X). A higher polyunsaturated fatty acid composition was found in lipids from animals fed high fat and high P/S diets and a higher monounsaturated fatty acid composition in lipids from animals fed low fat and low P/S diets. The diabetic state, compared to the normal state, was associated with a reduced content of polyunsaturated fatty acids, particularly in animals fed high P/S diets. The total body content of essential fatty acids was estimated to be lowest in diabetic animals fed low P/S diets.

Hypothesis 2

Differences in the content and the compostion of diet fat influenced the phospholipid content of the adipocyte plasma membrane (Chapter VII). Raising the fat content of the diet increased the content of phosphatidylethanolamine and reduced the content of sphingomyelin in both diabetic and control animals. High P/S diets were associated with a higher level of phosphatidylcholine in the adipocyte plasma membrane. When high fat diets were fed, the diabetic state was associated with a lower content of phosphatidylethanolamine and a higher content of phosphatidylinositol.

Hypothesis 3

In both the normal and diabetic states differences in the content and composition of diet fat influenced the fatty acyl composition of adipocyte membrane phospholipids (Chapters III, VI and VII). Although individual phospholipid classes responded to dietary fatty acid manipulation to different degrees, conclusions can be drawn. Feeding high P/S diets, compared to low P/S diets, significantly increased the polyunsaturated fatty acyl composition of membrane phospholipids (Chapters III, VI and VII). As the P/S ratio of the diet increased, the content of monounsaturated fatty acids in membrane phospholipids decreased (Chapters VI, VII and IX). High fat (20% w/w) diets were associated with a higher content of polyunsaturated fatty acids and a lower content of monounsaturated fatty acids in membrane phospholipids (Chapter VII). In membranes from diabetic animals a higher content of $C_{18:2(6)}$ and lower content of $C_{20:4(6)}$, total w-3 fatty acids and total monounsaturated fatty acids were observed (Chapters VI and VII). Compared to diet-matched controls the change in polyunsaturated fatty acids in the diabetic state was more dramatic in animals fed high P/S diets (Chapters III, VI and VII).

Hypothesis 4

Feeding high P/S diets, compared to low P/S diets, significantly improved insulin binding to adipocytes from control animals (Chapters II and V). Scatchard analysis suggested that the increase in binding was due to an increased number of available high affinity low capacity receptor sites (Chapters III and V). At most insulin concentrations, adipocytes from

diabetic animals bound more insulin than cells from control animals fed low P/S diets (Chapters III and IV), but the P/S ratio of the diets fed did not significantly influence insulin binding to adipocytes from diabetic animals (Chapters III and IV).

Hypothesis 5

Increasing the P/S ratio of the diet significantly improved insulin-stimulated glucose transport by control cells (Chapters V and VIII) and, at higher insulin concentrations, by diabetic cells (Chapter V). Reducing the fat content of the diet enhanced insulin stimulated glucose transport in control cells but reduced transport in diabetic cells.

Hypothesis 6

Feeding high P/S diets improved insulin stimulated glucose oxidation by diabetic cells and glucose incorporation into lipids by both diabetic and control cells (Chapters IV and VIII). When fed as part of a high fat (20% w/w) diet, the P/S ratio did not appear to influence insulin stimulated glucose oxidation by control cells (Chapter IV). However, when low fat diets were compared, adipocytes from control animals fed low P/S diets demonstrated an increased capacity for insulin stimulated glucose oxidation and glucose incorporation into lipids than cells from animals fed high P/S diets (Chapter VIII).

Hypothesis 7

A direct positive relationship was demonstrated between diet P/S ratio, membrane phospholipid fatty acyl composition and insulin binding to the adipocyte plasma membrane.

The data presented in this thesis support previous work *in vitro* (Ginsberg et al., 1981; Grundfeld et al., 1981; Gould et al., 1982) that insulin binding and insulin action in the adipocyte are sensitive to the lipid composition of the plasma membrane. The results extend current *in vivo* understanding of insulin receptor mediated functions by; 1. feeding diets representative of segments of the North American population, with respect to both the content and composition of diet fat; and 2. utilizing a diabetic model characterized by cellular resistance to insulin stimulated action.

The diabetic state was found to alter the fatty acyl composition of the major phospholipids of the adipocyte membrane (Chapters III, VI and V) and the stored lipids in the adipocyte (Chapter X), similar to that reported in other membranes (Faas & Carter, 1980) and tissues (Worcester et al., 1979; Van Doormaal et al., 1984). Unexpectedly, however, the diabetic state was found to modify the effect of diet on membrane phospholipid composition (Chapters III, VI and VIII). The reason for this alteration is not clear. The time frame selected for the present study involved feeding diets for three weeks before diabetes induction and another three weeks post-streptozotocin injection. It is conceivable that this may not have been a sufficiently long enough period to normalize, by diet fat, the effects of disease-induced changes occuring in membrane composition. Potential difficulties, are encountered when studying untreated diabetic animals for longer periods of time as animals will lose weight and the mortality rate will increase. The effect of age when begun on diet treatment may have also contributed to the effect of diet on diabetic cells. For example, in Chapter III, where a slightly older animal (140g vs 50g) was studied and diets were fed for a shorter period of time, the effect of diabetes on the monounsaturated and $C_{18:2(6)}$ fatty acyl content of the membrane were not as extensive. Alternatively, the modest alteration in P/S ratios in the diets fed may not have been sufficient enough to alter diabetes-associated membrane changes. Future studies could potentially design diets aimed at correcting or attenuating changes induced by diabetes. The present study was not designed to achieve this objective.

The reduced $\Delta 6$ and $\Delta 9$ desaturase products anticipated in the diabetic state were observed in Chapter VI, VII and X. In general, feeding high P/S diets magnified the increase in $C_{18:2(6)}$ and feeding a low P/S diet amplified the decrease observed in monounsaturated fatty acids. The role of these defects in determining insulin action can only be hypothesized from the results of the present experiments, where the high P/S diet clearly improved insulin action in adipocytes (Chapters IV, V and VIII). Desaturase enzymes are insulin sensitive (Brenner, 1974) and insulin therapy has been demonstrated in some tissues to partially normalize the desaturase defect (Faas & Carter, 1980). It would, therefore, be interesting to

design a study where insulin is administered to the diabetic animals to identify the role of membrane alterations in normalizing cellular insulin action in the diabetic state. This is a logical direction to follow as diabetic patients with serum glucose levels similar to the animals used in the experiments reported in this thesis, would in most situations, be receiving exogenous insulin.

Insulin binding to control adipocytes was clearly improved by increasing the P/S ratio of the diet (Chapters III, V and IX). A direct relationship was established between diet-induced membrane alterations, particularily in phosphatidylethanolamine, and insulin binding. The improved binding produced in animals by feeding high P/S diets may be involved in the improved insulin stimulated glucose transport and intracellular glucose metabolism observed when high P/S diets were fed (Chapters IV and VII). Despite clear defects in insulin stimulated cellular action in adipocytes from diabetic animals, the effect of diet on insulin binding was not significant (Chapters III and V) . The reason for this observation can only be hypothesized. The alterations produced in membranes from diabetic animals by feeding high P/S diets may not have been great enough or of the right type to produce the desired alterations in binding. The diabetic state was associated with a reduce content of phosphatidylethanolamine and a fatty acid compostion of this phospholipid that would be expected to reduce insulin binding (Chapter VII). To address this question one might, using a diabetic animals model, repeat the experiment described in Chapter IX where diets providing the ten different P/S ratios were fed. Moreover, additional factors such as the short period of time (three weeks) diabetic animals were fed the diets and the degree of diabetes produced in the animals may alter insulin binding. Both of these questions could be easily answered in follow up experiments.

Although the existance of a binding defect in human diabetes, is at present, controversial (Sinha et al., 1987; Arner, 1987), it has been reported that the streptozotocin diabetic rat, compared to control animals, demonstrates enhanced insulin binding (Schoenle et al., 1977). The results of this thesis indicate that this may not be true under all conditions as streptozotocin diabetic rats, compared to control animals fed high P/S diets, bound the same

or less insulin than control cells.

Diet fat clearly influenced the relationship between insulin binding and glucose transport (Chapter V). Although diabetic animals, compared to control animals, clearly transported less glucose per unit insulin bound, feeding a high P/S diet to both control and diabetic animals increased the amount of glucose transported per insulin bound (Chapter V). This observation suggests the compostion of diet fat could influence the function of insulin post receptor binding. This is a logical prediction as the plasma membrane provides the interface for the initial contact of the hormone with the cell and has been postulated to be important in generating the cellular actions of insulin (Jarett & Kriechle, 1984; Larner et al., 1982; Haystead & Hardie, 1986; Begum et al.,1981). Results presented in this thesis demonstrate, that in the normal state, the plasma membrane is an integral determinant of insulin binding (Chapter IX) and in the short-term, feeding a high P/S diet to diabetic animals significantly improved insulin mediated functions (Chapters IV, V and VIII). It would therefore be logical to follow up these observations by measuring the effect of diet fat on some of the proposed membrane generated mediators of insulin action such as phosphorylation of the tyrosine kinase associated with the β-subunit of the insulin receptor.

Recent evidence suggests that w-3 fatty acids may be important in both insulin action (Strolien et al., 1987) and pancreatic insulin release (Turk et al., 1987). Although the levels of w-3 fatty acids supplemented in the diets fed in the experiments reported in this thesis were low, the content and composition of diet fat and the diabetic state influenced the content of w-3 fatty acids in the structural (Chapter VII) and stored lipids (Chapter X) of the adipocyte. In light of the recent interest in very long chain w-3 fatty acids derived from fish oils in the treatment and prevention of various diseases, an experiment could be designed to determine if insulin action is further improved by supplementing diets with a source of these fatty acids.

Current dietary recommendations for both the general population and individuals with diabetes recommend that the level of fat be reduced and a portion of saturated fats be replaced with polyunsaturated fats (American Diabetes Association, 1987; Kisselbach &

Schectman, 1988). Although the physiological implications of these dietary modifications remain to be determined, this thesis suggests that increasing the polyunsaturated content of the diet improves epididymal adipocyte insulin binding and action and may prove beneficial in reducing insulin resistance associated with the diabetic state. In extrapolating the results of this thesis to man one must consider that modifications in dietary fat content and composition will also alter the protein and carbohydrate components of the diet, which have been demonstrated to influence cellular insulin action (Kergoat et al., 1987; Kettelhut et al., 1985). This thesis generates sufficient evidence to justify the design of a human feeding protocol to further elucidate the role of localized changes in membrane composition in modulating insulin action within cells and the importance of these changes in the management of the diabetic state.

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APPENDIX

Table A-1. Marker enzyme activity in cell fractions.

	Enzyme Specific Activity (n=4)	
	5'-Nucleotidase	Succinate Dehydrogenase
Homogenate	.769±0.03	.680±.04
Plasma membrane	6.46±0.04	1.47±.12
Mitochondria	.66±0.17	13.70±.12
Nuclear pellet	2.14±0.23	3.62±.26

Marker enzyme activities mean±S.E. were determined from control animals fed the P/S=2.0 (n=2) and P/S=0.25 (n=2). 5'-nucleotidase actiity is expressed as μmoles phosphorus hydrolyzed/mg protein/hour at 37°C by method of Goldfine et al. (1977). Succinate dehydrogenase activity is expressed as μmoles of para-iodonitrozolium violet reduced/mg protein/hour at 37°C by method of Kun & Abood (1949).

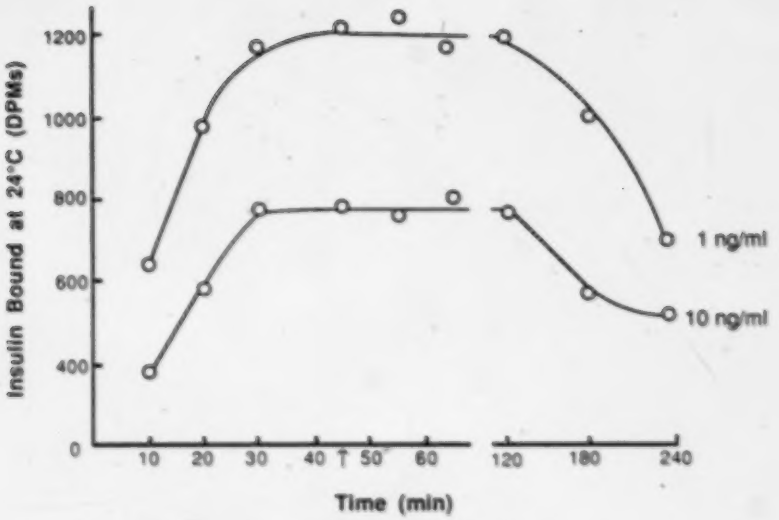

Figure A-1. Insulin binding to adipocytes over time at 24°C.

The amount of ^{125}I-insulin bound over time at two insulin concentrations (1 ng/ml and 10 ng/ml). Maximum binding (n=2 samples/3 replicate) was achieved by 30 mins. and maintained until 120 mins. To ensure maximum binding at 24°C, cells were incubated with insulin for 45 mins.

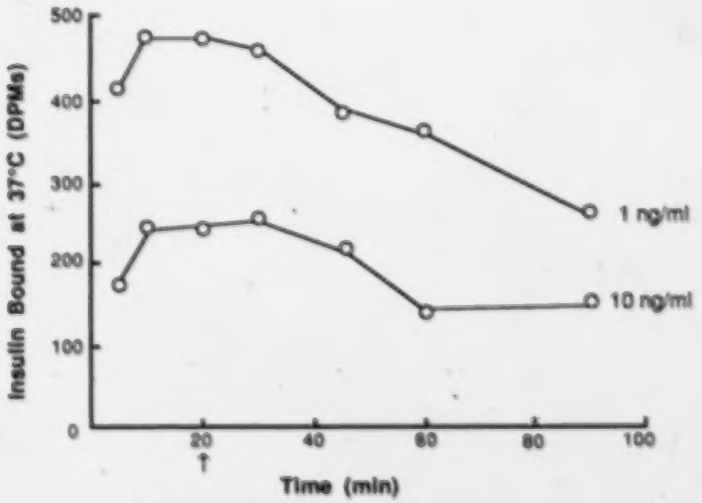

Figure A-2. Insulin binding to adipocytes over time at 37°C.

The amount of ^{125}I-insulin bound over time at two insulin concentrations (1 ng/ml and 10 ng/ml). Maximum binding was achieved by 10 mins. and maintained until 30 mins. To ensure maximum binding at 36°C, cells were incubated with insulin for 20 mins.

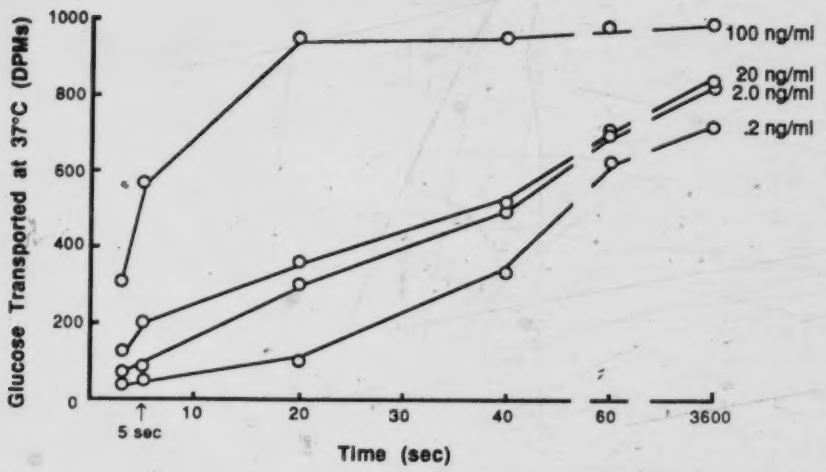

Figure A-3. Adipocyte glucose transport over time a 37°C.

The amount of 3-0-^{14}C-methyl-D-glucose transported (n=2 samples/3 replicates) by adipocytes at four insulin concentrations (.2, 2, 20 and 100 ng/ml insulin) at a glucose concentration of .5 mm. To ensure that measurements were in the linear portion of the uptake curve glucose transport assay was conducted at 5 secs.

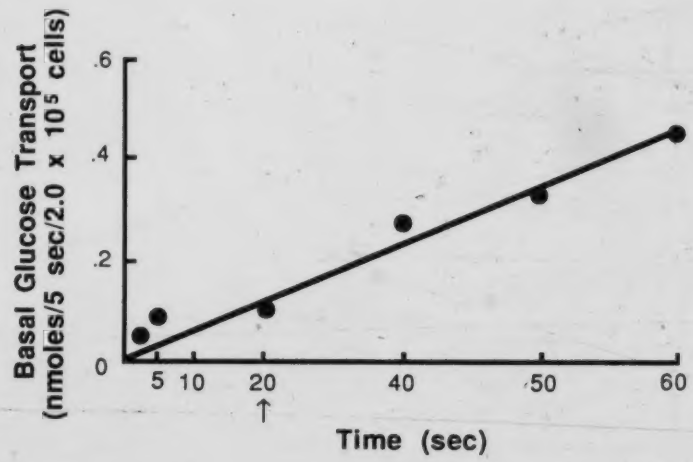

Figure A-4.Basal glucose transport over time at 37°C.

The amount of 3-0-^{14}C-methyl-D-glucose transported (n=2 samples/3 replicates) by adipocytes in the absence of insulin. Basal glucose uptake over the first minute was linear. As measurements at 5 secs tended to overestimate uptake, basal glucose measurements were conducted at 20 secs and divided by 4 to determine uptake at 5 secs.